

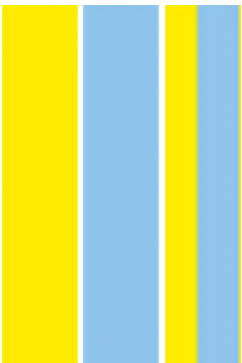
DOUTORAMENTO

BIOLOGIA BÁSICA E APLICADA

Biological and behavioral predictors for fear recall and generalization

Bruno José Moraes

D
2025



Biological and behavioral predictors for fear recall and generalization

Bruno José Rother Rocha de Moraes

BRUNO JOSÉ ROTHER ROCHA DE MORAES

BIOLOGICAL AND BEHAVIORAL PREDICTORS FOR FEAR RECALL AND GENERALIZATION

Tese de Candidatura ao grau de Doutor

Programa *Graduado em Áreas da Biologia Básica e Aplicada*
(GABBA)

Submetida a:

Instituto de Ciências Biomédicas de Abel Salazar

Faculdade de Ciências da Universidade do Porto

Faculdade de Medicina da Universidade do Porto

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Declaração de Honra

Declaro que a presente tese é de minha autoria e não foi utilizada previamente noutro curso ou unidade curricular, desta ou de outra instituição. As referências a outros autores (afirmações, ideias, pensamentos) respeitam escrupulosamente as regras da atribuição, e encontram-se devidamente indicadas no texto e nas referências bibliográficas, de acordo com as normas de referenciação. Tenho consciência de que a prática de plágio e auto-plágio constitui um ilícito académico.

Setúbal, 15 de Setembro, 2025

O doutorando



(Bruno José Moraes)

List of Publications

The following published original research manuscript was presented in this thesis:

Moraes, B.J., Hardt, O., Specific behaviors during auditory fear conditioning and post-synaptic expression of AMPA receptors in the basolateral amygdala predict inter-individual differences in fear generalization in male rats, *Learn. Mem.*, **30**, 74-84, 2023; doi:10.1101/lm.053612.122

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Para os meus pais

Margitta Rother Rocha de Moraes (1956-2006)

José Adriano Rocha de Moraes (1949-2022)

*a quem o Conhecimento não chegou a tempo
de permitir que estivessem presentes.*

Für meine Eltern

Margitta Rother Rocha de Moraes (1956-2006)

José Adriano Rocha de Moraes (1949-2022)

*denen das Wissen nicht rechtzeitig zugekommen ist,
um ihnen die Teilnahme zu ermöglichen.*

To my parents

Margitta Rother Rocha de Moraes (1956-2006)

José Adriano Rocha de Moraes (1949-2022)

*to whom Knowledge did not arrive in time
to allow them to be present.*

Recordações e agradecimentos

Toda a aventura deste doutoramento foi um sem-número de peripécias que começaram em 2010, logo após a conclusão do mestrado em bioquímica médica na FCUL, com uma sugestão do agora casal Carvalho-Bandarra (os meus bons amigos Sara e Daniel), e que terminam agora, em 2025, à moda antiga (os doutoramentos de hoje são muito mais breves), após 15 anos. Estas palavras serão por isso acompanhadas por um breve apontamento biográfico, talvez até mais para mim que para os leitores da tese — para me recordar de tudo isto quando me encontrar na situação dos ratinhos de mais de 20 meses da Figura 3.2.2E, e as minhas espinhas dendríticas já não sobreviverem mais de 40% no espaço de 4 meses. São, de facto, muitos anos e nem todos foram passados a trabalhar no doutoramento nem na tese. Na verdade, o trabalho experimental que deu origem a este manuscrito foi obtido integralmente no período que passei no Canadá, na McGill University, de Março de 2012 a Agosto de 2013 (apesar de ter depois continuado o doutoramento na Escócia, entre Setembro de 2013 e Dezembro de 2014, mas falarei desse período mais adiante).

O processo de entrevistas decorreu, para mim, no dia 13 de Junho (o dia em nasceu o poeta Fernando Pessoa, agora que penso nisso) de 2010, com um júri composto pela falecida Professora Doutora Maria de Sousa, mentora da criação do GABBA, e os Professores Doutores Alexandre do Carmo e João Relvas, o último dos quais veio a tornar-se meu co-orientador. Tive a felicidade de ser escolhido para a 14^a edição juntamente com outros



UNIVERSIDADE DO PORTO

PROGRAMA DOUTORAL EM ÁREAS DA BIOLOGIA BÁSICA E APLICADA • GRADUATE PROGRAM IN AREAS OF BASIC AND APPLIED BIOLOGY

List of Students - 14th Edition GABBA 2010/2014

Name	Date of Birth	1st Degree	Final Mark	University
Bandarra, Daniel	1987	Biochemistry		U. Lisboa
Ferreira, Filipa	1986	Molecular and Cell Biology		U. Nova de Lisboa
Fontes, Mariana	1986	Molecular Biology and Genetics		U. Lisboa
Lindo, André	1985	Physics Engineering		U. Aveiro
Martins, André	1986	Molecular Biology and Genetics		U. Lisboa
Miranda, Pedro	1984	Molecular and Cell Biology		U. Nova de Lisboa
Moraes, Bruno	1985	Biochemistry		U. Lisboa
Neto, Filipa	1985	Medicine		U. Porto
Oliveira, Ana	1984	Biology		U. Aveiro
Pinto, Ricardo	1987	Biology		U. Porto
Silva, Milene	1986	Pathological, Cytological and Thanatological Anatomy		Instituto Politécnico do Porto
Tenente, Inês	1987	Molecular Biology and Genetics		U. Lisboa

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11 colegas e, por razões que, entretanto, começam a tornar-se história, por curiosidade, vou deixar-vos uma imagem da vinheta enviada pela nossa amiga e secretária (incansável) do GABBA, Catarina Carona (ver figura acima). Eu e os meus colegas passámos os primeiros meses de doutoramento (de Janeiro a Julho de 2011) com aulas na cidade do Porto até se iniciar a procura por um tema de tese e um laboratório onde desenvolver as nossas competências teóricas e experimentais. Foi um período incrível de aprendizagem, tanto científica como de vida, e deixou-me muitas memórias insubstituíveis. Quero agradecer-vos a todos, Daniel, Filipa Ferreira, Mariana, André Lindo, André Martins, Pedro, Filipa Neto, Ana Catarina, Ricardo, Milene e Inês, pelo crescimento conjunto para que contribuíram, e pedir-vos desculpa se, na minha forma de ser por vezes reservada, não vos acompanhei em mais aventuras.

Com a ajuda dos meus co-orientadores, e seguindo a minha vontade de aprender mais sobre a memória, segui uma sugestão do Dr. Rui Costa e rumei ao Canadá, à McGill University, em Março de 2012, onde fui aceite pelo Dr. Karim Nader e comecei a trabalhar no departamento de psicologia do Stewart Biology Building. A história da minha investigação lá é essencialmente a história desta tese, como mencionei acima. Conheci muitas pessoas que não só me ajudaram a integrar no meu primeiro trabalho mais independente como cientista, como me apoiaram na resolução de muitas dúvidas e incertezas inerentes ao estudo do cérebro. Foram elas a Paola Virginia Miguez Blanco, a Karine Gamache, o Carmelo Milo e o Shih-Dar Chang. Há mais uma pessoa neste grupo, mas como acabou por ter um lugar muito especial na minha vida científica, já falo dela. Aproveito também para mencionar o Bruno Fonseca, que conheci no edifício ao lado, num dia em que precisei de ajuda com uma PCR de um colega dele (o Carmelo tinha-me dito que havia um português no grupo). É, entretanto, uma amizade tão velha como este doutoramento, que não morre apesar da distância (ele ainda está no Canadá mas falamos com frequência pelo telefone). O Bruno, sendo mais velho e experiente que eu, foi um companheiro de vida e ciência em Montréal. Mais tarde, durante o surto de Covid-19, vivemos uma pequena aventura de criação de uma empresa, a PrimerGen, com o intuito de produzir um tratamento. Não deu frutos, mas foi um excelente ensinamento para mim sobre o ramo empresarial. O Bruno teve também a amabilidade de me convidar a participar num importante artigo que escreveu sobre o processo de síntese proteica associada aos TOP mRNAs (também com grande importância para a formação de memórias de longo prazo); foi um prazer contribuir para a revisão do artigo e uma honra poder figurar na lista de autores.

Infelizmente, a primeira pedra que encontrei no caminho decorreu da mudança de chefia do departamento, que resolveu impor aos estudantes visitantes da McGill University um período máximo de permanência de 1 ano. Com o trabalho engrenado e após vários meses de esforço para entrar no campo das neurociências, foi um duro golpe quando recebi uma

notificação dos serviços académicos para abandonar a instituição de acolhimento no espaço de 6 meses.

Pouco tempo antes tinha começado a trocar frequentemente impressões sobre artigos, procedimentos experimentais, psicologia e bioquímica com um pós-doutorado do grupo do Dr. Nader chamado Oliver Hardt. Encontrava-me com ele e com a Virginia no gabinete deles para um brainstorming sobre algum tema que nos interessasse. Neste período, acompanhou alguns dos meus protocolos bioquímicos e mostrou-se muitas vezes interessado no meu trabalho. Como é habitual na nossa carreira, o Dr. Hardt pretendia eventualmente deixar de ser pós-doutorado e formar o seu laboratório, e recebeu, em 2013, um convite do Dr. Richard Morris para ir trabalhar para a Universidade de Edimburgo. Tendo tido conhecimento da minha situação, e sendo que eu teria de abandonar a McGill até Agosto desse ano, o Dr. Hardt perguntou-me se eu gostaria de me juntar a ele (a Virginia também se juntou mais tarde). O Dr. Nader estava já bastante doente nesta altura e a minha saída adicional do seu laboratório deixava-me numa situação complicada de falta de mentor. Decidi aceitar. Fiz as malas e em Setembro de 2013 estava a procurar casa ao som de gaitas de foles.

O Dr. Hardt é o último elemento do grupo que mencionei acima. Eu sou o primeiro doutorando dele, e ele é hoje o meu único orientador oficial. O Oliver (esta deferência do “Dr. Hardt” está a custar-me muito) tem sido um orientador incansável. Duvido profundamente que muitos doutorandos tenham a sorte de poder chamar “amigo” a um orientador, e eu orgulho-me de o poder fazer. Eu não consigo expressar aqui o quanto lhe devo por este momento, porque não se trata de uma soma de eventos isolados que o justifiquem, mas de uma relação contínua de ensinamento e confiança nas minhas capacidades (em como eu iria conseguir terminar o doutoramento) que me sustentou ao longo de todos estes anos. E ainda temos trabalho à nossa espera. Obrigado, Oliver. Por tudo.

Estive com ele em Edimburgo entre Setembro de 2013 e Dezembro de 2014, altura em que a minha bolsa da FCT terminou e me vi obrigado a regressar a Portugal. Muitos se questionarão sobre os dados de Edimburgo, por que razão não figuram na tese. Bom, a resposta é simples: porque a vida não é linear e eu fui confrontado com um segundo obstáculo no meu doutoramento, mas desta vez intransponível. Enquanto no Canadá estudei a expressão dos receptores AMPA nas densidades pós-sinápticas da amígdala, no contexto de memórias associadas ao medo, na Escócia o contexto mudou para as memórias espaciais associadas também à amígdala, sim, mas sobretudo ao hipocampo e aos córtices infra- e paralímbico. Para isso treinei os meus ratos a encontrar uma plataforma submersa numa grande piscina com água opaca (a Morris water maze) e pretendia ver como é que isso mudava a expressão dos receptores AMPA nas áreas que mencionei. Infelizmente, o meu pai foi atropelado em Fevereiro de 2014 e durante o período em que estive

em Portugal para o ajudar, todos os cérebros que eu tinha recolhido ao longo de meses dos ratos que treinei na Morris water maze, e que esperavam guardados a -80 °C, tinham a ultra-estrutura totalmente alterada, como claro resultado de um processo de descongelamento e novo congelamento. Na minha ausência tinha havido uma mudança das amostras de todos os grupos de um freezer para outro novo; nunca saberei se foi neste processo ou se houve qualquer outra razão. O que importa é que estávamos em Julho de 2014, a poucos meses de terminar a minha bolsa e era-me impossível fazer a análise bioquímica que pretendia, uma vez que teria de treinar novos animais para o efeito. O último período em Edimburgo foi passado a tentar resumir os meus dados todos do Canadá a fim de compor uma história publicável. Em Janeiro de 2015 estava de volta a Portugal e precisei de uma pausa de tudo. Foi quando recebi um convite para integrar a estreia de uma peça de Natália Correia ("O Homúnculo") com o meu grupo de teatro de longa data, o Teatro Estúdio Fontenova.

Ao longo dos anos seguintes, entre 2015 e 2018, tentei de forma muito fragmentada prosseguir com o agrupamento dos meus dados e a construção da história que figuraria na minha tese, mas a falta de um meio científico estimulante não me permitiu essa recuperação. Neste período fui pai de uma menina, a Petra, agora já com nove anos, a quem agradeço a vivacidade e desafio constantes, bem como o ambiente transformador da vida que é a paternidade. A responsabilidade de uma criança a cargo conduziu-me a um trabalho precário numa fábrica de produção de produtos do tomate durante uns meses, bem como a um ano como professor de música numa escola primária. Depois, em 2018, achei que, com a minha filha mais crescida e para ter qualquer hipótese de terminar a tese, teria de voltar a ter um emprego científico. Candidatei-me a algumas bolsas no campo das neurociências e em resultado de uma aceitação, estive a trabalhar durante dois anos no Centro de Neurociências de Coimbra, com a Dr.^a Ana Cristina Rego e a Dr.^a Ildete Luísa Ferreira, na doença de Huntington. A ambas agradeço a confiança que depositaram em mim, apesar dos três anos fora da bancada. Não foi um período fácil, mas durante esse tempo consegui finalmente reunir os dados do Canadá e, apercebendo-me do efeito de generalização de medo muito diverso na população de ratos que utilizei, extraí deles um resultado experimental com algum interesse científico. Em 2020, contudo, teve início a pandemia de Covid-19, com as consequências conhecidas, e no fim desse ano, sem verbas suficientes no laboratório, fiquei sem bolsa e regressei à minha terra natal. Por fim, no início de 2021 e até Fevereiro de 2023, estive no laboratório do Dr. José Paulo Sardinha, no Instituto Superior Técnico, em Lisboa, a extrair compostos com actividade antioxidante de algas marinhas, com o intuito de os usar no tratamento de neuropatologias. Todas estas pessoas, sem o saberem, tiveram um papel importante no meu percurso, porque me permitiram regressar e manter vivo no meio científico, bem como garantir o necessário financiamento

subjacente às minhas responsabilidades parentais, não deixando de fazer o que gosto. A todos eles estou agradecido pelas oportunidades que me deram.

Conheci a Sofia em Setembro de 2021. Um pouco como o Big Bang terá iniciado distraidamente o Universo, o encontro dos nossos caminhos modificou a minha vida a todos os níveis e gerou em mim, ainda que tardiamente, um novo propósito no meu caminho que eu já não acreditava ser possível, mas sobretudo uma felicidade que já não sentia há muito. É difícil descrever o que temos e talvez por isso seja tão natural e belo. Como posso explicar a alguém a sensação permanente de um jogo de equipa que nunca tem, nem nunca precisa de quaisquer pausas? Quantas pessoas poderão usar a sua experiência pessoal de vida para compreender que algumas coisas que só se vêem nos filmes, como casais que mutuamente completam as frases um do outro, que fazem os mesmos comentários perante as mesmas situações, que tomam as mesmas decisões sem diálogo prévio, que tudo isso pode mesmo acontecer? Tal como o Oliver, a Sofia nunca desistiu de mim nem de me fazer acreditar que terminar este longo doutoramento era possível. A ela devo a publicação do meu artigo como primeiro autor em 2023: fomos incansáveis vezes para a biblioteca da Escola Superior de Educação do Instituto Politécnico de Setúbal para eu conseguir a concentração necessária para o terminar e enviar ao Oliver. A publicação do artigo foi uma verdadeira alavanca de Arquimedes. Para além de todos os dias desde que nos conhecemos, todas as músicas que existem no mundo e ela tem em si (e tanto que ela canta, cantando ou sem cantar), todas as trocas de ideias, curiosidades e aprendizagens em conjunto, científicas ou de outro teor, é o verdadeiro conforto do companheirismo diário que me completa e impele para cada novo dia. Obrigado por existires. Obrigado por todas as aventuras. Obrigado por tudo o que ainda vamos concretizar juntos.

A escrita da tese, depois, teve a preciosa ajuda da família da Sofia, nomeadamente da Cristina Vilar e do José Picareta Jorge, os seus pais, que me cederam temporariamente uma casa para me isolar e me apoiaram de inúmeras formas com tudo quanto estive ao seu alcance. A presença do avô Valdemar, do irmão Daniel Vilar, e da sua companheira Ana Roda, tem sido igualmente uma constante. É muitas vezes nos pequenos detalhes que se geram as oportunidades e eu não menosprezo nenhum minuto passado com eles: tenho aprendido imenso com todos vocês. Obrigado, Cristina e Zé e a toda a minha nova família.

Ao longo de todos estes anos, diversas pessoas têm partilhado comigo as suas vidas, enriquecendo-me e mantendo-me atento e ligado ao que me rodeia. Mas sobretudo uma, a mais longa amizade e que melhor me conhece e melhor conseguiria retratar-me numa biografia, é o Ricardo Vilhena. Um pouco como na didascália inicial de “Felizmente Há Luar!”, de Luís de Sttau-Monteiro, em que se diz que embora o General Gomes Freire de Andrade nunca apareça está sempre presente — podemos ter de requerer a tua presença,

Ricardo, mas tu nunca me falhaste com nada e sempre tiveste a amizade pronta nas mãos para me dar a qualquer hora e em qualquer lugar. Espero conseguir sempre corresponder-te.

À Maria Odília Rocha deixo o meu sentido agradecimento por todo o apoio que me deu ao longo da vida. Esteve presente em alguns dos momentos mais difíceis, mas tem igualmente partilhado a sua presença em encontros de grande alegria, comigo, com a Sofia e com a Petra.

Desejo, por fim, agradecer aos meus co-orientadores, o Dr. Rui Costa e o Dr. João Relvas, que não só me apoiaram na escolha do laboratório onde desenvolvi o trabalho experimental presente na tese, como foram indirectamente responsáveis pelas pessoas maravilhosas que conheci, e estiveram comigo até ao último momento, a escassos dias da entrega. Muito obrigado a ambos. Professor João, espero ter estado, de alguma forma, à altura da escolha que fizeram em Junho de 2010.

...

A 10 de Maio de 2022 perdi o meu pai. Nessa noite, antes de o saber, um homem idoso com uma mala de viagem bateu-me no vidro do carro. A Sofia e eu íamos sair e detivemo-nos a fim de saber o que o senhor pretendia. O senhor perguntou-me se eu era filho do Sr. Moraes e, ao responder-lhe afirmativamente, disse-me que o meu pai falava com muito orgulho em mim e no doutoramento que eu estava ainda a realizar. Eu tentei corrigir o senhor, colocando a expressão no presente: “fala”, disse-lhe eu — porque o meu pai estava nesse momento no hospital. O senhor então sorriu e despediu-se desejando-me tudo de bom para o futuro, pegou na mala de viagem e arrastou-a em direcção ao fim da rua, onde acabou por desaparecer na distância. Uns minutos depois recebi o telefonema do hospital.

Nem o meu pai, nem a minha mãe, que já partiu há muitos anos, vão poder ver esta etapa da minha vida. E por isso, mais do que para todas as pessoas vivas que ainda vão ter essa oportunidade, esta tese é para eles.

Obrigado por tudo o que fizeram por mim e que me fez chegar a este momento.

Es muss sein.

Recollections and acknowledgments

The whole adventure of this PhD was a series of countless events that began in 2010, shortly after completing my master's degree in medical biochemistry at FCUL, with a suggestion from the now married couple Carvalhal-Bandarra (my good friends Sara and Daniel), and which has now ended, in 2025, in an old-fashioned way (today's doctorates are much shorter), after 15 years. These words will therefore be accompanied by a brief biographical note, perhaps more for myself than for the readers of the thesis — to remind me of all this when I find myself in the situation of those mice over 20 months old in Figure 3.2.2E, and my dendritic spines no longer survive more than 40% within 4 months. It has indeed been many years, and not all of them were spent working on my doctorate or thesis. In fact, the experimental work that gave rise to this manuscript was obtained entirely during the period I spent in Canada, at McGill University, from March 2012 to August 2013 (although I then continued my PhD in Scotland between September 2013 and December 2014, but I will talk about that period later).

The interview process took place on June 13 (the birthday of the poet Fernando Pessoa, now that I think about it) of 2010, with a jury composed of the late Professor Maria de Sousa, mentor of the creation of GABBA, and Professors Alexandre do Carmo and João Relvas, the latter of whom became my co-advisor. I was fortunate to be chosen for the 14th edition along with 11 other colleagues and, for reasons that are now becoming history, out of curiosity, I will leave you with an image of the vignette sent by our friend and (tireless) secretary of GABBA, Catarina Carona (see figure above within the Portuguese text). My colleagues and I spent the first months of our doctorate (from January to July 2011) attending classes in the city of Porto until we began searching for a thesis topic and a laboratory where we could develop our theoretical and experimental skills. It was an incredible period of learning, both scientifically and in terms of life experience, and it left me with many irreplaceable memories. I want to thank you all, Daniel, Filipa Ferreira, Mariana, André Lindo, André Martins, Pedro, Filipa Neto, Ana Catarina, Ricardo, Milene, and Inês, for the joint personal development you contributed to, and apologize if, in my sometimes reserved nature, I did not accompany you on more adventures.

With the help of my co-advisors, and following my desire to learn more about memory, I took up a suggestion from Dr. Rui Costa and headed to Canada, to McGill University, in March 2012, where I was accepted by Dr. Karim Nader and began working in the psychology department of the Stewart Biology Building. The story of my research there is essentially the story of this thesis, as I mentioned above. I met many people who not only helped me integrate into my first more independent work as a scientist, but also supported me in resolving many doubts and uncertainties inherent to the study of the brain. They were

Paola Virginia Miguez Blanco, Karine Gamache, Carmelo Milo, and Shih-Dar Chang. There is one more person in this group, but as he ended up having a very special place in my scientific life, I will talk about him later. I would also like to mention Bruno Fonseca, whom I met in the building next door one day when I needed help with a PCR from a colleague of his (Carmelo had told me that there was a Portuguese person in the group). It is, however, a friendship as old as this PhD, which has not died despite the distance (he is still in Canada, but we talk frequently on the phone). Bruno, being older and more experienced than me, was a companion in life and science in Montreal. Later, during the Covid-19 outbreak, we embarked on a small adventure to set up a company, PrimerGen, with the aim of producing a treatment. It didn't come to fruition, but it was an excellent lesson for me about the business world. Bruno was also kind enough to invite me to participate in an important article he wrote about the process of protein synthesis associated with TOP mRNAs (also of great importance for the formation of long-term memories); it was a pleasure to contribute to the review of the article and an honor to be included in the list of authors.

Unfortunately, the first obstacle I encountered was the change in department leadership, which decided to impose a maximum stay of one year on visiting students at McGill University. With my work well underway and after several months of effort to enter the field of neuroscience, it was a hard blow when I received a notification from the academic services to leave my host institution within six months.

Shortly before, I had begun to frequently exchange ideas about articles, experimental procedures, psychology, and biochemistry with a postdoctoral fellow from Dr. Nader's group named Oliver Hardt. I would meet with him and Virginia in their office to brainstorm about topics that interested us. During this period, he followed some of my biochemical protocols and often showed interest in my work. As is common in our career, Dr. Hardt eventually wanted to leave his postdoctoral position and start his own laboratory, and in 2013 he received an invitation from Dr. Richard Morris to work at the University of Edinburgh. Aware of my situation, and knowing that I would have to leave McGill by August of that year, Dr. Hardt asked me if I would like to join him (Virginia also joined later). Dr. Nader was already quite ill at this point, and my additional departure from his lab left me in a complicated situation without a mentor. I decided to accept. I packed my bags, and in September 2013, I was looking for a house to the sound of bagpipes.

Dr. Hardt is the last member of the group I mentioned above. I became his first doctoral student, and he is now my only official advisor. Oliver (this deference to "Dr. Hardt" is costing me a lot) has been a tireless advisor. I seriously doubt that many doctoral students are lucky enough to be able to call their advisor a "friend," and I am proud to be able to do so. I cannot express here how much I owe him for this moment, because it is not a series of isolated events that justify it, but a continuous relationship of teaching and trust in my abilities (in

how I would be able to finish my PhD) that has sustained me throughout all these years. And we still have work ahead of us. Thank you, Oliver. For everything.

I was with him in Edinburgh between September 2013 and December 2014, when my FCT scholarship ended and I was forced to return to Portugal. Many will wonder about the data from Edinburgh, and why it does not appear in the thesis. Well, the answer is simple: because life is not linear and I was faced with a second obstacle in my PhD, but this time it was insurmountable. While in Canada I studied the expression of AMPA receptors in the postsynaptic densities of the amygdala, in the context of memories associated with fear, in Scotland the context changed to spatial memories also associated with the amygdala, yes, but above all with the hippocampus and the infra- and paralimbic cortices. To do this, I trained my rats to find a submerged platform in a large pool of opaque water (the Morris water maze) and wanted to see how this changed the expression of AMPA receptors in the areas I mentioned. Unfortunately, my father was hit by a car in February 2014, and during the period I was in Portugal to help him recover, all the brain specimens I had collected over months from the rats I trained in the Morris water maze, which were stored at -80°C, had their ultrastructure completely altered, as a clear result of a process of thawing and refreezing. In my absence, the samples from all groups had been moved from one freezer to another; I will never know if it was during this process or if there was some other reason. What matters is that it was July 2014, a few months before my scholarship ended, and it was impossible for me to do the biochemical analysis I had intended, since I would have to train new animals for that purpose. I spent my last period in Edinburgh trying to summarize all my data from Canada in order to compose a publishable story. In January 2015, I was back in Portugal and needed a break from everything. That was when I received an invitation to join the premiere of a play by Natália Correia ("O Homúnculo") with my long-standing theater group, Teatro Estúdio Fontenova.

Over the following years, between 2015 and 2018, I tried in a very fragmented way to continue grouping my data and constructing the story that would feature in my thesis, but the lack of a stimulating scientific environment prevented me from making this recovery. During this period, I became the father of a girl, Petra, now nine years old, whom I thank for her liveliness and constant challenges, as well as for the life-changing environment that is parenthood. The responsibility of caring for a child led me to take on precarious work in a tomato product factory for a few months, as well as a year as a music teacher in a primary school. Then, in 2018, I realized that, as my daughter was growing up and in order to have any chance of finishing my thesis, I would have to return to scientific work. I applied for some scholarships in the field of neuroscience and, as a result of being accepted in one, I worked for two years at the Coimbra Neuroscience Center with Dr. Ana Cristina Rego and Dr. Ildete Luísa Ferreira on Huntington's disease. I thank both of them for the trust they

placed in me, despite my three years away from the bench. It was not an easy period, but during that time I finally managed to gather the data from Canada and, realizing the very different generalization effect of fear in the rat population I used, I extracted from them an experimental result of some scientific interest.

In 2020, however, the Covid-19 pandemic began, with its well-known consequences, and at the end of that year, without sufficient funds in the laboratory, I lost my scholarship and returned to my homeland. Finally, from early 2021 until February 2023, I was in Dr. José Paulo Sardinha's laboratory at the Instituto Superior Técnico in Lisbon, extracting compounds with antioxidant activity from marine seaweeds, with the aim of using them in the treatment of neuropathologies. All these people, without knowing it, played an important role in my journey, because they allowed me to return and remain active in the scientific community, as well as securing the necessary funding to support my parental responsibilities, without giving up on what I loved to do. I am grateful to all of them for the opportunities they gave me.

I met Sofia in September 2021. A bit like the Big Bang may have distractedly started the Universe, the meeting of our paths changed my life on all levels and generated in me, albeit belatedly, a new purpose in my path that I no longer believed possible, but above all a happiness that I had not felt for a long time. It is difficult to describe what we have, and perhaps that is why it is so natural and beautiful. How can I explain to someone the permanent feeling of a team game that never has, nor ever needs, any breaks? How many people can use their personal life experience to understand that some things you only see in movies, like couples who finish each other's sentences, who make the same comments in the same situations, who make the same decisions without prior discussion, that all of this can really happen? Like Oliver, Sofia never gave up on me nor on making me believe that finishing this long PhD was possible. I owe her the publication of my article as first author in 2023: we went tirelessly to the library of the School of Education of the Polytechnic Institute of Setúbal so that I could get the concentration I needed to finish it and send it to Oliver. The publication of the article was a real Archimedes lever. In addition to every single day since we met, all the music that exists in the world and that she carries within her (and she sings so much, whether really singing or not), all the exchanges of ideas, curiosities, and learning together, scientific or otherwise, it is the true comfort of daily companionship that completes me and propels me into each new day. Thank you for existing. Thank you for all the adventures. Thank you for everything we will still achieve together.

The writing of the thesis then had the invaluable help of Sofia's family, namely Cristina Vilar and José Picareta Jorge, her parents, who temporarily gave me a house to isolate myself in and supported me in countless ways with everything within their reach. The presence of her grandfather Valdemar, her brother Daniel Vilar, and his partner Ana Roda

has also been a constant. It is often in the small details that opportunities arise, and I do not take any minute spent with them for granted: I have learned so much from all of you. Thank you, Cristina and Zé, and all my new family.

Over the years, many people have shared their lives with me, enriching me and keeping me alert and connected to my surroundings. But above all, the longest friendship, the person who knows me best and who could best portray me in a biography, is Ricardo Vilhena. A bit like in the opening caption of Luís de Sttau-Monteiro's *Felizmente Há Luar!* theatre play, which states at the beginning that although General Gomes Freire de Andrade never appears, he is always present — we may have to ask for your presence, Ricardo, but you have never failed me in anything and have always had your friendship ready to give me at any time and in any place. I hope I can always reciprocate.

To Maria Odília Rocha, I offer my heartfelt thanks for all the support she has given me throughout my life. She was there during some of the most difficult moments, but she has also shared her presence in moments of great joy, with me, Sofia, and Petra.

Finally, I would like to thank my co-advisors, Dr. Rui Costa and Dr. João Relvas, who not only supported me in choosing the laboratory where I carried out the experimental work presented in this thesis, but were also indirectly responsible for the wonderful people I met, and who were with me until the very last moment, just a few days before the deadline. Thank you both very much. Professor João, I hope I have somehow lived up to the choice you made in June 2010.

...

On May 10, 2022, I lost my father. That night, before I knew it, an elderly man with a suitcase knocked on my car window. Sofia and I were about to leave and stopped to find out what the man wanted. He asked me if I was Mr. Moraes' son, and when I answered yes, he told me that my father spoke very proudly of me and the doctorate I was still working on. I tried to correct him, putting the expression in the present tense: “talks,” I said, because my father was in the hospital at that moment. The man then smiled and said goodbye, wishing me all the best for the future, picked up his suitcase, and dragged it toward the end of the street, where he eventually disappeared into the distance. A few minutes later, I received a call from the hospital.

Neither my father nor my mother, who passed away many years ago, will be able to see this stage of my life. And so, more than for all the living people who will still have that opportunity, this thesis is for them.

Thank you for everything you have done for me and that has brought me to this moment.
Es muss sein.

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1 Thesis synopses

1.1 Abstract

Auditory fear conditioning in rats is a widely used method to study learning, memory, and emotional responding. Despite procedural standardizations and optimizations, there is substantial inter-individual variability in fear expression during test, notably in terms of fear expressed towards the testing context alone. To better understand which factors could explain this variation between subjects, we here explored whether behavior during training and expression of AMPA receptors (AMPA receptors) after long-term memory formation in the amygdala could predict freezing during test. We studied outbred male rats and found strong variation in fear generalization to a different context. Hierarchical clustering of these data identified two distinct groups of subjects that independently correlated with a specific pattern of behaviors expressed during initial training (i.e., rearing and freezing). The extent of fear generalization correlated positively with post-synaptic expression of GluA1-containing AMPA receptors in the basolateral nucleus of the amygdala. Our data thus identify candidate behavioral and molecular predictors of fear generalization that may inform our understanding of some anxiety-related disorders, such as posttraumatic stress disorder (PTSD), that are characterized by over-generalized fear.

1.2 Resumo

O condicionamento clássico auditivo ao medo em ratos é um método amplamente utilizado para estudar a aprendizagem, a memória e a resposta emocional. Apesar de ser um procedimento experimentalmente padronizado e otimizado, existe uma variabilidade inter-individual substancial na expressão do medo pelos ratos durante o teste à memória associativa, 24 h pós-condicionamento, nomeadamente em termos do medo expresso em relação à caixa onde os animais são testados, mas antes do teste sonoro. Com o objectivo de compreender melhor os factores que poderiam explicar essa variação entre indivíduos, decidimos explorar se o comportamento durante o treino e a expressão dos receptores AMPA (AMPA_Rs) na amígdala após o teste e com a associação de longo prazo formada, poderiam prever o comportamento de *freezing* (congelamento motor) observado durante o teste. Estudámos ratos machos não consanguíneos e confirmámos uma forte variação na generalização do medo num ambiente diferente (caixa de teste) do ambiente de treino. A análise estatística desses dados por agrupamento hierárquico identificou dois grupos distintos de indivíduos que se correlacionavam de forma independente com um padrão específico de comportamentos expressos durante o treino inicial: *rearing* (postura exploratória assente nos membros posteriores) e *freezing*. A extensão do comportamento de generalização do medo apresentou uma correlação positiva com a expressão pós-sináptica de AMPA_Rs contendo a subunidade GluA1 no núcleo basolateral (BL) da amígdala. Os nossos dados identificam, assim, candidatos a preditores comportamentais e moleculares para a generalização de memórias associadas ao medo que podem ser úteis à compreensão de algumas patologias relacionadas com a ansiedade, como é o caso da perturbação de stress pós-traumático (PSPT), caracterizada por comportamentos de medo generalizado excessivo.

2 List of abbreviations

2P	Two-photon
4.1N	Band 4.1-like protein 1 (<i>formerly Neuronal protein 4.1</i>)
A1	Primary auditory (<i>cortical area</i>)
ACC	Anterior cingulate cortex
AFC	Auditory fear conditioning
AIS	Axon initial segment
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPA	AMPA receptor
AP	Action potential
APV	DL-2-amino-5-phosphonovalerate
ARP2/3	Actin-related protein 2/3 complex
ATD	Amino-terminal domain
Ba	Basal (<i>amygdala nucleus</i>)
bAP	Backpropagating AP
BL	Basolateral (<i>amygdala nucleus</i>)
BNST	Bed nucleus of the stria terminalis
CA1	Cornu Ammonis area 1 (<i>hippocampal subfield</i>)
CA3	Cornu Ammonis area 3 (<i>hippocampal subfield</i>)
Ca _v	Voltage-dependent calcium channel
Ce	Central (<i>amygdala nucleus</i>)
CFC	Contextual fear conditioning
ChR2	Channelrhodopsin-2
CICR	Calcium-induced calcium release
CNIH	Cornichon homolog protein
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	Central nervous system
CREB	cAMP response-element binding protein
CS	Conditioned / conditioning stimulus
CTD	Carboxyl-terminal domain
DG	Dentate gyrus (<i>hippocampal subfield</i>)
dSpike	Dendritic spike
E_{AMPA}	Nernst potential for AMPA ion (<i>also: reversal potential</i>)
EPSC	Excitatory postsynaptic current
EPSP	Excitatory postsynaptic potential
EPSP _{soma}	Somatic EPSP

EPSP _{spine}	Spine EPSP
ER	Endoplasmic reticulum
GABA	Gamma-Aminobutyric Acid
GluA1-4	Glutamate receptor 1-4 (<i>also: AMPA-selective glutamate receptor 1</i>)
GluN2A/2B	Glutamate receptor ionotropic (<i>NMDA subtypes 2A and 2B</i>)
GRIP/ABP	GRIP / AMPA receptor-binding protein
GRIP	Glutamate receptor interacting protein
HCN	Hyperpolarization-activated cation channel
HFS	High-frequency stimulation
HRP	Horseradish peroxidase
I	Total ion current
I-V	Current-voltage
i_{AMPA}	Single-channel AMPA current
IP ₃	Inositol-1,4,5-trisphosphate
IP ₃ R	IP ₃ receptor
IPSP	Inhibitory postsynaptic potential
K _{Ca}	Calcium-dependent potassium channel
KO	Knockout
K _v	Voltage-dependent potassium channel
La	Lateral (<i>amygdala nucleus</i>)
L-VGCC	L-type voltage-gated calcium channel
L2/3	Layer II/III
L3	Layer III
L5	Layer V
L5b	Layer V-b
LBD	Ligand-binding domain
LFS	Low-frequency stimulation
LTP	Long-term potentiation
M1	Primary motor (<i>cortical area</i>)
MAP1A	Microtubule-associated protein 1A
MAP2	Microtubule-associated protein 2
miR-92a	MicroRNA 92a
mRNA	Messenger RNA
MSN	Medium spiny neuron
N	Number of channels
NA	Nucleus accumbens
Na _v	Voltage-dependent sodium channel

NMD	Nonsense-mediated decay
NMDA	N-methyl-D-aspartic acid
NMDAR	NMDA receptor
NRF-2	Nuclear factor erythroid 2-related factor 2
NSF	N-ethylmaleimide-sensitive factor
OFC	Orbitofrontal cortex
PDZ	PSD95-Dlg1-zo-1 (<i>protein domain</i>)
PFC	Prefrontal cortex
PICK1	Protein interacting with C kinase 1
PKA	Protein kinase A
PKC	Protein kinase C
PKM ζ	Protein kinase C zeta type
PNS	Peripheral nervous system
P_o	Open probability
pS295	phospho-Serine 295 (<i>protein PTM</i>)
pS831	phospho-Serine 831 (<i>protein PTM</i>)
pS845	phospho-Serine 845 (<i>protein PTM</i>)
PSD	Postsynaptic density
PSD95	Postsynaptic density protein 95
PTM	Posttranslational modification
PTP	Post-tetanic potentiation
PTSD	Posttraumatic stress disorder
Q/R	Glutamine / Arginine (<i>amino acids</i>)
RE	Recycling endosome
RNA	Ribonucleic acid
RyR	Ryanodine receptor
S1	Primary somatosensory (<i>cortical area</i>)
S2	Secondary somatosensory (<i>cortical area</i>)
SAP97	Synapse-associated protein 97
SLM	Stratum lacunosum-moleculare (<i>hippocampal layer</i>)
SNV	Single-nucleotide variant
SO	Stratum oriens (<i>hippocampal layer</i>)
SP	Stratum pyramidale (<i>hippocampal layer</i>)
SP4	Specificity Protein 4 (<i>transcription factor</i>)
SR	Stratum radiatum (<i>hippocampal layer</i>)
STED	Stimulated-emission depletion
STP	Short-term plasticity

TARP	Transmembrane AMPAR regulatory protein
TBI	Traumatic brain injury
UPF2	Regulator of nonsense transcripts 2 (<i>also: Up-frameshift suppressor</i>)
UPS	Ubiquitin-proteasome system
US	Unconditioned stimulus
V1	Primary visual (<i>cortical area</i>)
V_m	Membrane potential (<i>also: voltage</i>)

3 Introduction

3.1 Foreword

The following introduction will serve as a guide for the reader of the present thesis, who is not assumed to be a stranger to the field of Neuroscience. It will present an overview with discussion on some of the state-of-the-art of those themes *directly* involved in my studies which I found most relevant, namely AMPA receptors (AMPA_Rs) and behavioral aspects of learning and memory involving fear and the rat amygdala, in particular the basolateral (BL) nucleus (**Figure 3.1.1**), and some *not directly* involved in my studies, such as spine dynam-

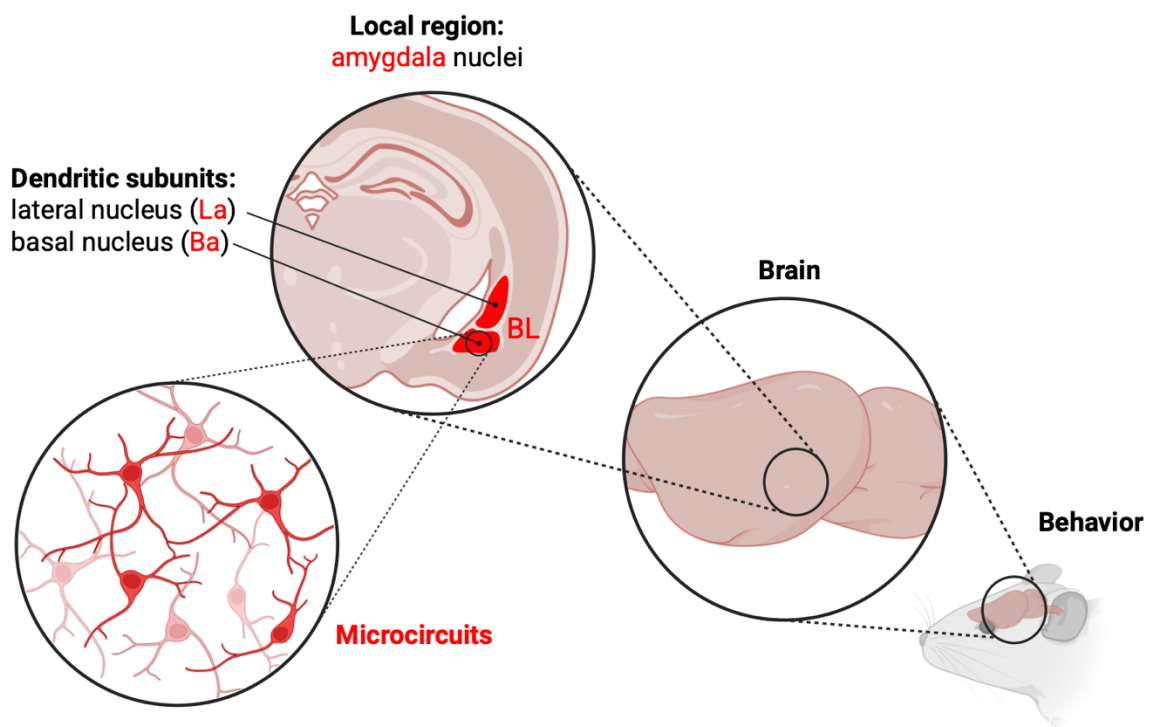


Figure 3.1.1. Simplified top-down organization of neural systems, from behavior to the amygdala microcircuitry. The organism behaves, which is coordinated by the brain's several local regions (here exemplified by a set of two amygdala nuclei), themselves composed of many neurons which perform computations within their dendritic subunits, e.g. the lateral (La) and the basal (Ba) nuclei, collectively known as the basolateral (BL) nuclei, using specialized patterns of connections called microcircuits.

ics, and electrophysiological considerations on the excitatory postsynaptic potential (EPSP) and related phenomena (e.g. long-term potentiation, or LTP) but that I still found inevitable to discuss, given the role ultimately played by AMPARs in them. Thus, while Section 3.2 discusses some technical aspects of neuronal dendrites and spine pruning in both standard and enriched housing conditions, as well as spine persistence within the context of fear learning, Section 3.3 more thoroughly expands on the history and current knowledge of

AMPA biochemistry and its involvement in fear memory processes. Section 3.4 discusses synaptic plasticity as a result of AMPAR synaptic expression, namely several forms of LTP, the involvement of dendrites and its spines, and how it all relates to memory persistence. Most basic research in the field has been conducted in hippocampal and neocortical pyramidal cells, and part of the data and discussion hereafter are based on these cells, but applying the findings to amygdala neurons, where possible.

3.2 Dendritic spines: the AMPARtance of being earnest¹

Where I present an overview of dendritic spine studies and their caveats, invoke their relevance for AMPARs, and wring out the literature for what is known on amygdala spines.

No two animals are truly alike, even clones accumulate different mutations and change, biochemically, in response to environmental stimuli throughout their lifespans. Within the human prefrontal cortex there is genetic mosaicism motivated by the accumulation of about 16 somatic single-nucleotide variants (SNVs) per year in an individual neuron (Luquette et al., 2022), affecting both enhancer and promoter regions, with potential for functional impact². Still, at the evolutionary macroscale, there is evidence for an interspecies neurodevelopmental plan: a direct comparison between mice (*Mus musculus*) and non-human primates (Rhesus macaque, *Macaca mulatta*) shows that neuron development occurs at similar rates for these species (Wildenberg et al., 2023), despite the fact that they diverged some 87 million years ago (Kumar et al., 2017). There is also evidence from studies on the zebrafish (*Danio rerio*) that neuron maturation might be relatively experience-independent (Barabási et al., 2022), i.e. many of the connections established during neurodevelopment do not significantly change through interactions of the organism with the surrounding environment. For organisms to thrive in nature, much of neurodevelopment must be programmed, making up the blueprint of our shared senses and nervous system functional regions. This evidently implies a high degree of precision and stability for interneural connections. Still, animals do learn and continuously transform connections to adapt behaviors to their surroundings, which is accomplished via many biological mechanisms, one of which is neuronal **spine pruning**.

¹ After Oscar Wilde, “The Importance of Being Earnest” (1895).

² Given these regions affect gene expression and therefore influence protein quality, and availability, which ultimately dictates changes in neuron function.

Despite their complex functions, dendritic spines are rather simple structures (**Figure 3.2.1**). They protrude from dendrite branches at densities ranging from 0.01 to 10 μm^{-1} , depending on neuron type, area location, animal maturation stage, plasticity requirement, and to be fair, technical involvement, given over the years different experimental techniques and resolution windows have been available. Spines are the sites of excitatory synaptic contact between neurons, harboring postsynaptic densities (PSDs; see Section 3.3), where

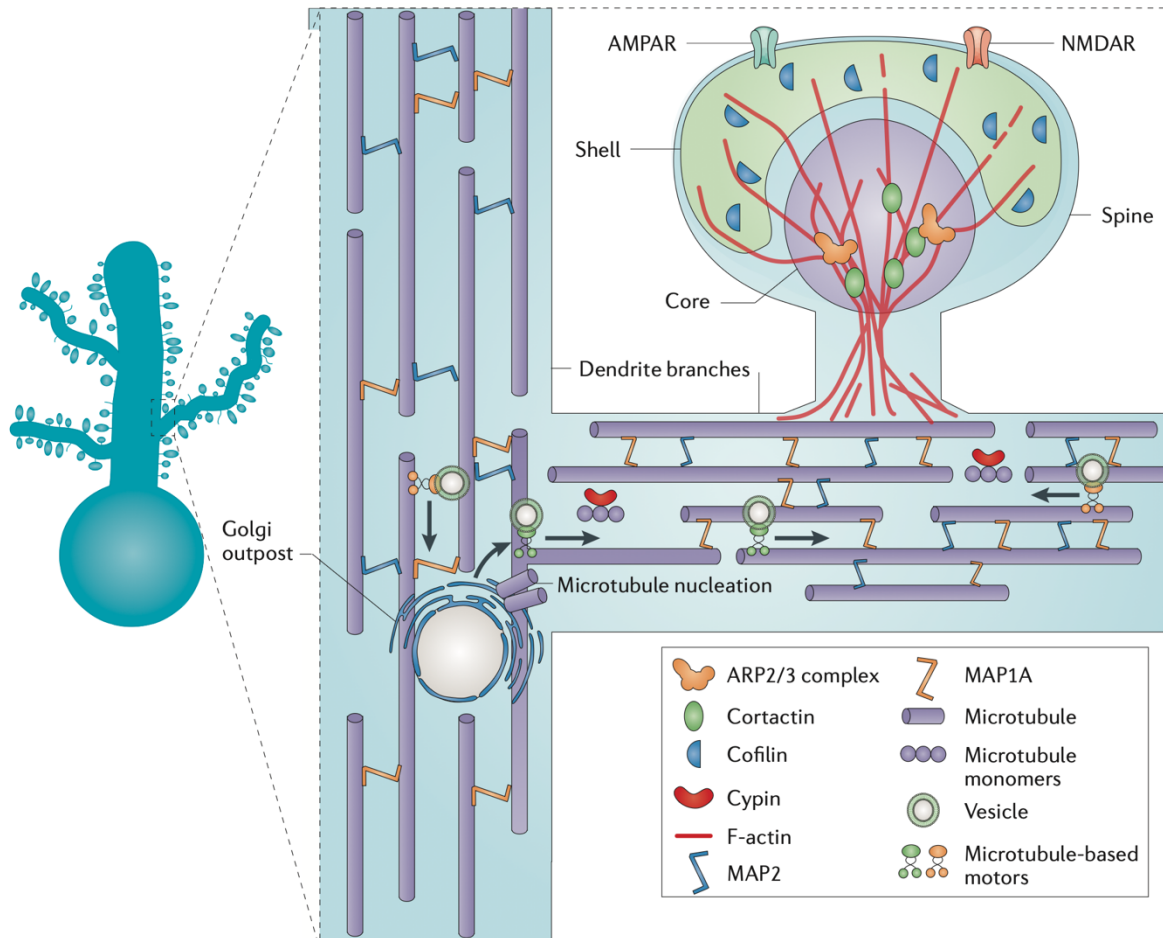


Figure 3.2.1. Overview of dendritic and spine structure. While the major building blocks of dendrites are microtubules, actin filaments handle the dynamical life of spines. Both cytoskeletal scaffolds are held up by protein connectors, namely microtubule-associated proteins (MAP1A, MAP2) and an actin-related protein (ARP2/3) complex, respectively. Specifically in spines, we find two regions: the central *core* is stabilized by ARP2/3 and Cortactin, while the outer *shell* is more dynamically shaped by Cofilin, harbors the postsynaptic densities (PSDs) seen in electron microphotographs, where AMPA receptors (AMPARs) are found, and makes up the characteristically mushroom-shaped aspect of mature spines. After Koleske (2013).

most of AMPA receptor (AMPAR) dynamics and function take place at the outer *shell*, and their active remodeling is under the control of the actin cytoskeleton and its associated proteins (Koleske, 2013). The main purpose of the spine apparatus seems to be biochemical compartmentalization, to allow for precise electrochemical signal point-to-point transduction between neurons, whereby the electrically provoked release of neurochemicals by the pre-synaptic neuron induces the continuation of the electrical signal on the postsynaptic neuron,

in the form of the excitatory postsynaptic potential (EPSP). Note that there is a major downscale of the voltage amplitude in this event sequence: the action potential (AP) arriving at the axon terminal is about 100 mV in magnitude, while a single spine transduces only about 10-30 mV of it (the spine EPSP; see Section 3.4), and due to heavy passive dendritic filtering³ only some 0.5-1 mV actually reach the postsynaptic soma (somatic EPSP) (G. J. Stuart & Spruston, 2015). Frequently overlooked, this downscale process is an important control point for the development of the neural code, thus requiring differential orchestration of many spines and synaptic contacts throughout dendritic arbors to integrate meaningfully at the cell body. AMPARs are at the center of this.

Classification of spine shape and the relation of the latter with its function have become increasingly complex over the years, thanks to technical improvement in imaging methods. Still, most authors will accept a minimal cutoff of three types of dendritic specializations: (a) thin protrusions extending up to 2 μm and usually forming no synapses are called *filopodia*, which are extremely dynamic, with turnover times as low as a few minutes, and mostly present during early developmental stages; in the event a filopodial protrusion evolves into a new spine, this may then either become (b) a *transient spine*, with a lifespan of less than 4 days, and a small or absent head volume, with less than 30% having synaptic contacts, or (c) a *persistent spine*, with a larger head volume of either stubby- or mushroom-type, almost always forming synapses, and with a lifespan above 8 days. I conducted an extensive literature meta-analysis (2002-2020) on the stability of pre-existent⁴ spines along the life of mice not subjected to any behavioral protocol (Attardo et al., 2015; Clark et al., 2018; Della Sala et al., 2016; Grutzendler et al., 2002; Gu et al., 2014; Holtmaat et al., 2005; Huang et al., 2020; S. K. Kim & Nabekura, 2011; Majewska et al., 2006; Mostany et al., 2013; Pfeiffer et al., 2018; Trachtenberg et al., 2002; Xu et al., 2007; Yang et al., 2009; Zuo, Lin, et al., 2005; Zuo, Yang, et al., 2005) (summarized in **Figure 3.2.2**). Most data collection on dendritic tree complexity and spine density was obtained from mice housed in groups of 2 or 3 animals inside standard cages, 12/12h light/dark cycles, with either gender mix or separation. A few other studies using complex housing have shown that environmental enrichment⁵, compared to standard housing, may increase both dendritic tree complexity and

³ Electrical compartmentalization provided by spine neck resistance is still under intense debate (Zecevic, 2023), with some arguing for high resistance values, which would isolate spines electrically (and further filter the EPSP between the spine head and the parent dendrite), while others find neck resistance to be low, thus having minimal influence on EPSP filtering. As with many other aspects of neuronal function, we will have to wait for further technical developments and reproducibility to resolve this important issue beyond doubt.

⁴ At the start of image recording, so that these are of either type (b) or (c).

⁵ Environmental enrichment, or complex housing, is not standardised but usually implies ≥ 4 animals per housing cage, itself bigger in size, with access to toys, ladders, several floors or levels. Objects may change identity or

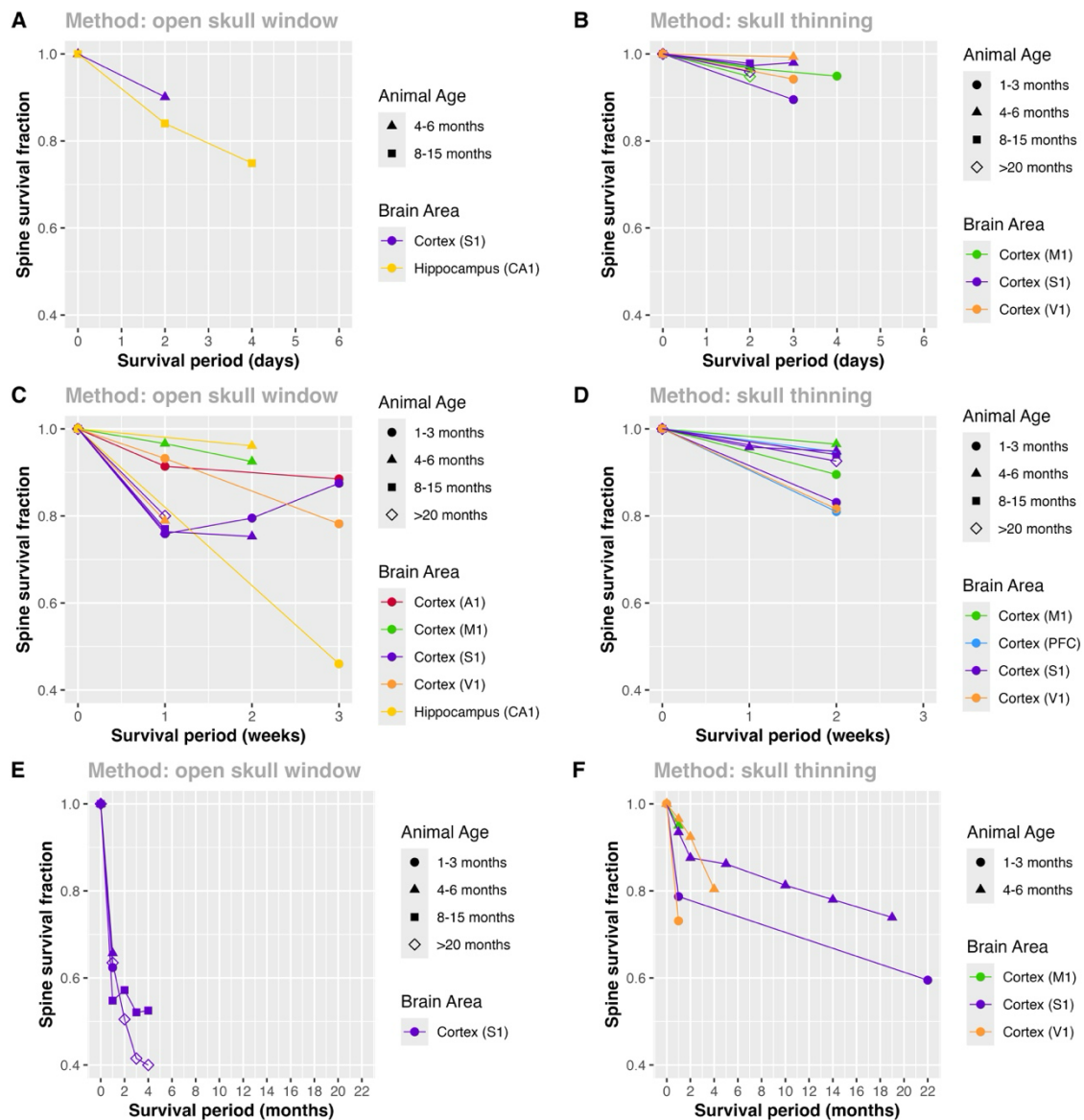


Figure 3.2.2. Dendritic spine survival across different mouse ages and brain areas. The plots show a summary of the data available from the literature (2002-2020; see main text) regarding the persistence of pre-existing dendritic spines over 1-6 days (**A, B**), 1-3 weeks (**C, D**) and 1-22 months (**E, F**) following first microscopic identification. Where more than one value was available from the literature for a particular set, the average was plotted. Animal ages were grouped into 1-3, 4-6, 8-15 and >20 months old at start of recording. Left and right column plots display results obtained with the two-photon open skull window, or skull thinning window technique, respectively. A 40% cutoff on the y-axis was chosen from the minimum reported value (plot E) to allow for better data visualization. Abbreviations: A1, primary auditory; M1, primary motor; PFC, prefrontal cortex; S1, primary somatosensory; V1, primary visual. Generated in R with packages *tidyverse* and *ggpubr*.

spine density of rat parietal (S2) cortical pyramidal neurons (Leggio et al., 2005), diabetic mouse hippocampal CA1 neurons (Beauquis et al., 2010), and PFC neurons (Ashokan et

location every day. Food access, albeit *ad lib.*, is frequently varied in taste and texture, and provided as indirect sources, such that animals must look for them or manipulate their surroundings in order to eat, much as they would have to do in natural environments.

al., 2018), as well as spine density in short-day impaired, white-footed mice' hippocampal CA1 and CA3 neurons (Workman et al., 2009). Still, neurons seem to react differently to housing conditions depending on brain area origin and function. For instance, while pyramidal neurons from both mouse hippocampus CA1 and dentate gyrus (DG) subfields show increased dendritic length under either daily exercise or enriched housing conditions (compared to controls housed in standard cages), primary motor (M1) cortical layer V (L5) neurons' and striatum spiny neurons' dendrites from the same mice show no differences (Faherty et al., 2003). Others found complex housing to increase spine density and dendritic length in rat *nucleus accumbens*' (NA) medium spiny neurons and in cortical S1 pyramidal neurons, but found no change in PFC pyramidal neurons' dendritic length, only spine density (Kolb et al., 2003), meaning dendritic arborization remained the same, albeit more populated by spines. More recently, research into the anterior cingulate cortex (ACC) actually found a time-dependent decrease in layer III (L3) apical and basal dendritic complexity, as well as both ACC L5 and dorsal agranular insular region of the orbitofrontal cortex (OFC) L3 basal dendrite spine density, following up to 16 days of environmental enrichment (Comeau et al., 2010). On the contrary, the same authors discovered that the 16-day period of housing enrichment increased complexity of basal dendrites in S1 L3 neurons. Of relevance to the present thesis, complex housing actually seems to buffer the effect of fear by reducing both spine density and stress-induced dendritic branching of Wistar rat stellate/pyramidal **amygdala** BL neurons⁶ (Ashokan et al., 2016). Furthermore, when subjected to contextual fear conditioning, 2 month old Sprague-Dawley rats that had been housed in enriched environments showed faster CS-US association learning, and better context discrimination during test than the control group, housed under standard conditions (Woodcock & Richardson, 2000). As the Comeau et al. (2010) study shows, time spent inside enriched contexts before dendrite and spine evaluation may be a factor to consider, as while S1 L3 dendrite complexity only increased following the 16-day enrichment period, not before, ACC basal L3 dendrite bifurcations already diminished by 4 days of exposure. Indeed, the above studies vary considerably in complex housing exposure: 1.5 weeks (Beauquis et al., 2010), 2 weeks (Ashokan et al., 2016), 3 weeks (Ashokan et al., 2018), 10-12 weeks (Leggio et al., 2005), 12 weeks (Workman et al., 2009), 12-14 weeks (Kolb et al., 2003) and 13-17 weeks (Faherty et al., 2003). The effect of housing conditions is irrefutable but further studies will be needed to fully understand the impact of environmental enrichment on dendrites and spine dynamics within the brain, and to relate them functionally.

⁶ The authors assessed fear impact by way of open arm exploration in the elevated plus maze and found that time exploring the arms equaled control levels if stressed animals had been previously housed with environment enrichment.

All data from **Figure 3.2.2** come from spines, not filopodia, but some of the studies following spines for less than a week (**Figure 3.2.2A-B**) do not show whether recorded spines are of the transient or persistent type: some studies recorded spines only for 2 or 4 days (Huang et al., 2020; Pfeiffer et al., 2018), which makes it impossible to say if these spines, depending on the stringency of spine identification criteria, were short-term, recent but durable, or already long-term older spines. Each datum represents the measured survival period from an initial population of spines for a particular age and brain region. Also, due to data scarcity, I have not split pyramidal neurons for cortical and hippocampal layers. Still, most cortical neuron dendrites were placed in L5, and most hippocampal spines are from CA1 *stratum oriens*⁷. As knowledge accumulates, it will be important to distinguish these source layers: pyramidal neurons are the most abundant type within the neocortex, with their dendrites differentially stretching along its six layers, but researchers found, e.g., that the density and dynamics of spines are significantly higher in L2/3 than in L5 in both 1 and 4 month old mice (Tjia et al., 2017), which may have an impact on conclusions taken from either layer. Importantly, all image acquisitions have been so far performed under anesthesia, so that appreciation of spine dynamics in fully awake animals is still to be achieved. Furthermore, 2P microscopy observation of spines has been carried out using one of two surgical procedures: (i) in the *open skull window* method there is the removal and substitution of part of the skull by a glass coverslip, allowing for optical measurements; (ii) in the *skull thinning* method the skull is thinned around the region of interest and the observations are made through this thinned area. Xu et al. (2007) measured spine dynamics via 2P microscopy using both methods and found that there is considerable turnover burden provided by extensive microglia and astrocyte activation within the first 1-2 months following open skull surgery, which is not present when the skull thinning technique is used. The brain area most frequently used for spine pruning studies included in the meta-analysis was S1 (**Figure 3.2.3**). It can be readily seen that, even considering just this one region, there is considerable dispersion when the open skull method is used, following spine stability for up to 4 months (**Figure 3.2.3A**, n = 7 studies) whereas when the skull thinning method is chosen (**Figure 3.2.3B**, n = 5 studies), even when spine stability is observed past 19-22 months, the dispersion is much smaller. It is also important to note that in cortical areas,

⁷ The CA1 subfield of the rodent hippocampus proper is composed of a sequence of multiple juxtaposed layers of cells. The whole structure of pyramidal neurons is vertically spread across these layers. The pyramidal cell bodies are located at the *stratum pyramidale* (SP) layer; dorsally to SP, the *stratum oriens* (SO) layer contains the pyramidal neuron's basal dendrites; ventrally to SP (towards the dentate gyrus), the *stratum radiatum* (SR) layer contains the proximal apical dendrites, whereas the *stratum lacunosum-moleculare* (SLM) contains the distal apical dendrites. This distinction is important given different dendrites of the pyramidal neuron receive different inputs from other brain regions, with their spines performing different functions (Mizuseki et al., 2011).

non-filopodial dendritic spines less than 4 days old are not sure to support stable *synaptic* contacts with other neurons (Knott et al., 2006), indicating that emergent spines search for presynaptic partners first, and take time to establish new connections. Now, spatial resolution using 2P microscopy not always allows for good discrimination between adjacent spines (Attardo et al., 2015), and the temporal spacing of image recordings cannot rule out the recurrence of spines at the same dendritic location, which may be classified as “stable”, whereas actually different spines have emerged and disappeared at the same spot over time.

Having introduced the main caveats of the meta-analysis, we may better appreciate the

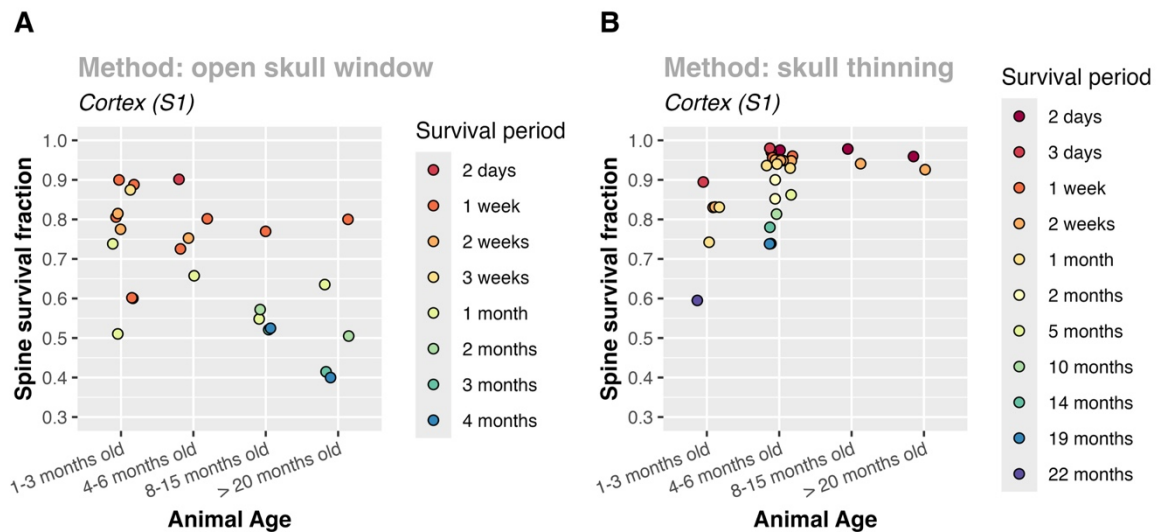


Figure 3.2.3. Spine survival in S1. The most abundant data come from the primary somatosensory (S1) cortex. The plots show spine survival fractions at several animal age intervals (1 to >20 months old) color coded for survival period, using either **(A)** the open skull window, or **(B)** the skull thinning methodologies for 2P microscopy. References for data in A: Trachtenberg *et al.* (2002), Holtmaat *et al.* (2005), Majewska *et al.* (2006) Xu *et al.* (2007), Kim & Nabekura (2011), Mostany *et al.* (2013), and Della Sala *et al.* (2016); references for data in B: Zuo, Lin *et al.* (2005), Zuo, Yang *et al.* (2005), Xu *et al.* (2007), Yang *et al.* (2009), and Huang *et al.* (2020). Generated in R with packages *tidyverse* and *ggpubr*.

results shown in **Figure 3.2.2**. The first observation is that no set of spines, regardless of animal age, brain area or even shortness of recording period (**Figure 3.2.2A-B**, a few days) remains forever, as they all decay over time. It is also clear from all survival periods, from short (days) to long (months), that spine survival fractions tend to fall at higher rates when animals are younger (< 1-3 months old) than when entering full adulthood (> 4-6 months old), irrespective of brain area, which is particularly visible for data obtained using the skull thinning method (**Figure 3.2.2B,D,F**). It is difficult to interpret the result of very long recordings, as animals age during that time and many physiological processes, including cognitive decay and age-dependent changes in spine elimination rates, take place. For instance, if

one starts recording at an age of 1 month, and then records for 22 months, by the end the animal will be 23 months old; this is the case of the S1 data in **Figure 3.2.2F**, where the survival fraction went from 76.6% at 1 month to 59.5% at 23 months of age (Zuo, Lin, et al., 2005). But the spine elimination rate is much lower at 4-6 months old, and so the same authors found that at 1 month of recording, 94.0% of the original spines were still present, and even 19 months later, at an age of ~23-25 months, the survival fraction was yet 73.9%. This example provides some biological support to our lifetime empirical observation that some memories formed during adolescence and early adulthood tend to be more numerous and stronger than those from the very early infancy. Indeed, findings akin to this observation have been reported in rodent models even for emotionally strong memories such as those acquired during fear-related learning. Relevant to our thesis, very young (~2 week old) mice trained in contextual fear conditioning show an unstable CS-US association that is gone around 7 days post-training, while that of ~1-2 month old mice lasts for at least 1 month (Akers et al., 2012). Also interesting is the finding that 18 day (~2 weeks) old Long-Evans rats show greater fear generalization following auditory-cue fear conditioning, than do 28 day (~1 month) old animals (J. W. Rudy & Pugh, 1996), meaning younger rats discriminate poorly between contexts. These two results from rodents are in line with the notion of higher spine pruning at 2 weeks (only 35% persistent S1 spines [≥ 8 days], not displayed in **Figure 3.2.2**), compared to that above 1 month of age or older (53.9-72.5%) (Holtmaat et al., 2005), which would ultimately explain why it is harder for younger animals to discriminate and, importantly, retain CS-US associations.

Above we saw that spine elimination rates are different at different animal ages, but this may also vary between brain regions, as one may appreciate by looking at **Figure 3.2.2**, albeit such conclusions will be hard to establish without further data. To illustrate this, one of the earliest studies using 2-photon (2P) microscopy to visualize mouse primary visual cortex (V1) layer 5 (L5) neuronal dendrites, evaluating spine stability over time, found that young animals (1 month old) displayed spine survival rates up to 73.1% over a period of 1 month (Grutzendler et al., 2002)⁸. The same year, another research group found lower values in primary somatosensory (S1) L5b barrel cortex neurons of young adult mice (1-2 months old), with only ~51% stable spines over the course of 1 month (Trachtenberg et al., 2002)⁹. Yet another group, a few years later, found a spine survival of 73.8% in similar conditions (Della Sala et al., 2016). These studies were performed using the two techniques

⁸ Overall *spine density* (number of spines per unit area) does not change appreciably in mice after they turn 1 month of age: it is the relative proportions of stable versus unstable (filopodia) spines which do, also dictating spine turnover rates (Holtmaat et al., 2005).

⁹ These authors also studied *dendritic branching* over a one-month period and found no appreciable changes.

described earlier, Grutzendler *et al.* using the skull thinning method (73.1% in V1, **Figure 3.2.2F**), and both Trachtenberg *et al.* and Della Sala *et al.* using the open skull window method (data average of 62.4% in S1, **Figure 3.2.2E**), which clearly provide very different results for the two brain areas, V1 and S1. These data are difficult to reconcile, especially given two other groups using the skull thinning method in S1 obtained similar survival figures to those of Grutzendler *et al.* for 1 month old animals in V1, and whose spines were also followed for 1 month (average 83.1% for 1-2 month old mice (Zuo, Lin, et al., 2005), and 74.2% for 1 month old mice (Zuo, Yang, et al., 2005), data average of 78.7% in S1, **Figure 3.2.2F**). It is true that besides the difference in critical developmental periods, with S1 developing earlier than V1 (Pedrosa et al., 2022), and accumulated understanding that rodents coordinate both whiskers and eye movements to scan their surroundings (Bergmann et al., 2022), the commitment of S1 and V1 sensory modalities within the poorly-enriched home cages may not be the same, given most often these cages still have richer textures and structural aspects (which engage S1) rather than diversity in visual cues (which engage V1). This may undoubtedly contribute to spine survival disparities reported among research papers.

More recent technological developments led to the possibility of live spine pruning assessment in deeper brain regions. One of the brain areas mostly studied for its relationship to learning and memory, anatomically close to the amygdala, is the hippocampus proper. Using the same 2P microscopy approach with a chronic open skull window into the dorsal hippocampus, one group provided the first study on the CA1 *stratum radiatum* layer of dendritic spines in the adult mouse (6-7 months old (Gu et al., 2014)). These authors found a population of 96.1% stable spines over a period of 16 days, with 4-day recording windows. Quite strikingly, another study using adult mice (2.5-3 months old) suggested a complete turnover of hippocampal basal (SO) CA1 neuron spines, with virtually no stable spines (0%) over a period of about 1 month based on mathematical models applied to imaging data to account for overestimation produced by 2P microscopy (Attardo et al., 2015); still, objectively, they reported 46% of persistent spines over the course of 22 days (3-day recording windows). A more recent study suggests more moderate spine turnover rates, with stable spines reducing to 74.9% (measured with 2P microscopy) or 60.8% (with 2P-STED¹⁰) also in basal (SO) hippocampal neurons from adult (4-12 months old) animals, recorded within just 4 days (Pfeiffer et al., 2018). The latter are not as long-term as the Attardo *et al.* data

¹⁰ Stimulated-emission depletion (STED) microscopy, together with 2P microscopy, provides an upgrade on spine resolution (Attardo et al., 2015). These authors studied the consequences of using 2P alone or in conjunction with STED and found the latter to improve resolution of two spines by a factor of about nine. Still, research into further resolution enhancements on 2P-STED microscopy is underway (Bancelin et al., 2021).

but clearly build on a resolution gain, using STED coupled to 2P microscopy. Albeit these studies are just a limited set, it may be that spines in different dendritic locations along CA1 pyramidal neurons (SR vs SO) have different dynamics. Overall, spine survival fraction half-lives seem to be lower in the hippocampus than in cortical areas (**Figure 3.2.2A,C**), which Attardo *et al.* justify with recourse to memory system reorganization theory¹¹ (Moscovitch & Gilboa, 2022), according to which, among other mechanisms, some memories initially reliant on the hippocampus slowly become independent of it, while being recoded into neocortical patterns, a process which can take about a month. Still, experimental reproduction, increased image resolution, and time extension of these protocols are necessary to support such conclusions.

Much of the abovementioned fragmented knowledge accumulated for both neocortical and hippocampal areas is, unfortunately, totally lacking for the **amygdala**. At present, not much is known about dendritic spine stability on any of the several amygdala neuron types and nuclei: no studies have been conducted so far to directly evaluate the permanence of spines during normal home cage exploration or following some form of environmental enrichment. One recent report, using 2P microscopy, imaged BL nuclei neurons¹² in acute slices from mice aged ~2 weeks old and ~1 month old to determine average spine motility (measured every 5 min for 1 hour), and found that motility decreased with age from ~0.02 $\mu\text{m}/\text{min}$ to ~0.01 $\mu\text{m}/\text{min}$ (Young et al., 2020), which is an early indication that BL spine pruning may decrease throughout mouse development as well. Determination of spine density, however, is far from established. Golgi-Cox impregnated slices from ~2 months old mice BL neurons¹² showed an average dendritic spine density of ~0.40 μm^{-1} , which increased following auditory fear conditioning in standard housing conditions (Heinrichs et al., 2013), but actually decreased below basal levels under housing enrichment with chronic immobilization stress (2 h/day) (Ashokan et al., 2016), although the latter group reported spine densities of ~0.66 μm^{-1} . Accordingly, animals with high-anxiety profiles display BL pyramidal neurons with more branched dendrites and higher spine densities than less anxious animals, but these other authors actually determined an average spine density of ~1.4 μm^{-1} (Adamec et al., 2012), which is 2-3 times higher than those from the other two reports. This may be the result of different “spine” definitions, which may include any of the three options given above (filopodia, transient, and persistent spines). One could argue, as is often the case when few data are available, that amygdala pyramidal neurons are probably

¹¹ Formerly the standard model of systems (or memory) consolidation. An alternative, multiple-trace theory, is less stringent on hippocampal independency, listing some memories (e.g. episodic) as hippocampus-dependent (Frankland & Bontempi, 2005).

¹² The authors didn't specify which BL neurons: pyramidal-like or non-pyramidal.

not very different from those found in other brain areas, and even between different species. However, a research group compared dendritic arbors of L and Ce amygdala nuclei' pyramidal-like neurons from rats and monkeys (Rhesus macaque, *Macaca mulatta*) and found that while dendritic spine densities were similar in Ce nucleus cells between these species, they were considerably higher (22%) in rat L nucleus neurons (Morgan & Amaral, 2014). This result may reflect different evolutionary adaptations of primates and rats. Also, dendritic tree length and soma volume are quite larger in both of macaque's nuclei (up to 250% for tree length), and spine length is about 20% longer in the Rhesus Ce nucleus. Pyramidal cells from areas expected to be more similar, such as the motor, somatosensory and visuo-temporal neocortices, despite showing equivalent spine densities along dendrites, have spines with different neck lengths and head size (Ballesteros-Yáñez et al., 2006). And even in the same animals, at 0.5-1 months of age, CA1 spine length (~1.30 μm) has been shown to be shorter than that of BL spines (1.52-1.44 μm) (Young et al., 2020). All these differences contribute disparities in dendritic integration, ultimately influencing how easily the neuron reaches AP threshold, and attest that it is not wise to compare neurons only by morphological class. Indeed, many computational models now point to a strict dependence of AMPAR dynamics on dendritic spine geometry (Bell et al., 2023; Simon et al., 2014), which makes it all the more important to relate AMPAR expression to both spine density and architecture, as well as developmental stage. For example, when two groups of Sprague-Dawley rats aged ~1 month old (juvenile) and ~3 months old (adult) were subjected to contextual fear conditioning (via 5 min of open area exploration plus 1 h on top of a small pedestal), similarly to other studies, the juveniles displayed no recall (no freezing) during test 7 days post-conditioning, as well as no significant changes in amygdala¹³ synaptic protein expression and posttranslational modifications. But adult rats had ~5 times increased synaptic GluA2, ~1.75 times increased GluA1-pS845, ~2 times increased PSD95 and PSD95-pS295, and a 50% decrease in PKM ζ (Zanca et al., 2019). These figures, obtained via subcellular fractionation and western blotting procedures, do not reflect change *per synapse*, but rather total synaptic values normalized to a non-conditioned, control condition. Still, given the relationship of the data with developmental stage (adults vs juvenile), and based on analysis of several other brain areas (**Figure 3.2.2**), it is quite possible they represent decreased dendritic spine elimination rates in the adults (i.e. increased numbers of persistent spines), with more room for stable increases in AMPAR subunits and other synaptic protein partners. Interestingly, this being a task not directly involving the amygdala, Zanca *et al.* saw no significant changes in adult amygdala synaptic proteins only 1 day after conditioning (but they did see changes in the dorsal hippocampus); yet, in an auditory fear conditioning setting,

¹³ The authors also evaluated synaptic proteins from the dorsal hippocampus.

such as the one used in our studies, and for which the amygdala is essential, ~2 month old rats did express stress-related increases in synaptic GluA1 (Moraes & Hardt, 2023). Therefore, it will be very important that future studies address the relationship between AMPAR expression and dendritic spine persistence.

3.3 The AMPA (R)eason

Where I briefly explore the history conducting to the identification of AMPARs, how they contribute to neuron excitability, their subunit distribution among the two main types of neuron in the rat BL nucleus, and how this influences behavior.

Although it had been available for over ten years, electron microscopy was only first applied to cells in the mid 1940s (Porter et al., 1945). This technique was going to have a profound influence in neuroscience, as in the 1950s it helped to definitely prove Santiago Ramón y Cajal's staining observations of neuron individuality, summarized in his *neuron doctrine* (Ramón y Cajal, 1899), as opposed to Golgi's "reticular theory". Sandy Palay and George Gray were certainly some of its pioneers in our field, having used electron microscopy to work out the architecture of neuronal cellular components such as synaptic vesicles (Palay & Palade, 1955), and quickly including Cajal's dendritic **spines** (Palay, 1958) (see Section 3.2) and **synapses** (Gray, 1959). Parallel to these developments in the cellular biology of nervous tissue was the notion that communication between neurons was fundamentally *electrochemical*. Chemical neurotransmission was worked out by Dale and Loewi at the beginning of the 20th century (Dale, 1914; Loewi, 1921). Also, that living matter conducted electricity was already known for a long time (Galvani et al., 1791; Volta, 1800), and the work of Charles Sherrington helped establish Cajal's doctrine and the first modern notions and importance of electrochemical *integration* in neuronal networks (Sherrington, 1906). Intracellular recordings from neurons were first performed in the late 1930s (Cole & Curtis, 1939; Hodgkin & Huxley, 1939), but the pipettes inserted into axons from the giant squid had tips with widths on the 100-140 μm range (D. G. Stuart & Brownstone, 2011), still far from what would be necessary to study mammalian neurons: to give the reader an idea, the average soma diameter of a CA1 pyramidal cell is 21 μm (G. Stuart et al., 2016). Sharp microelectrodes (0.5-1.0 μm) were only first introduced around 1950 (Ling & Gerard, 1949; Nastuk & Hodgkin, 1950), with the first intracellular recordings mostly taken from the cat spinal cord motoneuron *soma*. At that time, only very seldomly were these recordings made from large dendritic trunks, but never from small dendritic branches, much less directly from spines, as it was technically unfeasible — the diameter of a CA1 pyramidal dendrite is around 1-3 μm , with spine volumes of 0.004-0.6 μm^3 , and spine necks ranging 40-500 nm wide (G. Stuart et al., 2016). Indeed, the contributions from these neuron structures were

either still mostly ignored or only the subject of speculation, and the majority of research focused on the soma as the origin of neuron integration and axonal firing (the reader may consult Hodgkin and Huxley's groundbreaking papers, including the one in which they proposed their influential model of action potential generation in the *axon* (Hodgkin & Huxley, 1952)). It was assumed at the time that excitation at distal dendritic branches could not spread effectively to the soma, and thus had little or no contribution to integration there (a further ironic break from Cajal's prediction of "(...) the great physiological role played by the spines" (Ramón y Cajal, 1904), alluding to his expectation on the important role of dendritic trees). From a pronouncedly different perspective, and over the course of fifteen years of theoretical work (1959-1974), Wilfrid Rall provided evidence that distal dendritic input could not only theoretically reach the soma, but was essential for neuronal computations, delivering the first analytical solutions to simulations of excitatory postsynaptic potentials (EPSPs) measured at the soma, but which resulted from proximal and distal dendritic inputs, as well as from passive dendritic properties (Rall, 1962). His *shape-index plot*, proposed in 1967, provided a theoretical relation between the somatically measured EPSPs and the location of the inputs generating them (Rall et al., 1967). Rall achieved this by using experimentally observed miniature EPSPs, which are naturally elicited by neurons (there is no applied current), and comparing their shapes with his mathematical theory. He had to wait until 1983 for experimental confirmation of this relationship, achieved via simultaneous monitorization of EPSP generation and neuronal staining with horseradish peroxidase (HRP) to observe input location (Redman & Walmsley, 1983). This was perhaps the first major triumph of the emerging field of computational neuroscience. During this period of waiting, and together with John Rinzel, Rall also correctly predicted general features of steady-state (Rall & Rinzel, 1973) and transient (Rinzel & Rall, 1974) branch input spreading of dendritic *distally* injected currents, which are still being biologically, and computationally, elucidated to this day. It was also during this period that Neher and Sakmann developed the much needed *patch-clamp* technique (Neher & Sakmann, 1976). Eventually, in the 1990s, the first simultaneous patch-clamp recordings from the soma and dendrites of neurons were made using brain slices from rat neocortical layer V pyramidal cells (G. J. Stuart et al., 1993). **AMPA receptors** (AMPA receptors) had just been freshly cloned.

When an experimenter records from a single neuron, the interest usually falls on the membrane potential (V_m) changes measured with the electrodes. However, because the experimenter is unable to see the membrane-embedded proteins adjacent to the electrode tip, it is impossible to determine which channels or receptors are contributing to the electrical properties of V_m (namely its graphical shape), unless some pharmacological or staining procedure (chemical, optical, etc.) is implemented along with the recordings (such was the case with the ionic nature of the central EPSP until the 1990s). One important feature, given

synaptic transmission is both electrical and chemical — and which might also help identifying the proteins involved in V_m production — was the nature of the neurotransmitter used for communication. During the 1950s, while the abovementioned breakthroughs were being made, interest also arose around peripherally (PNS) identified acetylcholine and monoamines as potential neurotransmitters in the CNS. These, however, failed to mimic the very fast kinetics observed in central neurons. On the other hand, high concentrations of some amino acids (such as **L-glutamate** and L-aspartate) were found throughout the mammalian brain, and their exogenous application to the cortical surface could induce convulsions and depression (Lodge, 2009). At the entry of the 1960s, using microelectrophoresis, the group of J.C. Watkins successfully showed that spinal neurons could be excited with these amino acids (Curtis et al., 1960), which started a several-decades search for the receptors through which they exerted their actions. These **glutamate receptors** are presently classified, based on the identification of 18 genes, as *ionotropic glutamate receptors* of the NMDA, kainate, delta (δ) and **AMPA** (formerly *quisqualate*) families (Lodge, 2009; Traynelis et al., 2010). Interestingly, the first cloning of a glutamate receptor subunit (Gundersen et al., 1984; Hollmann et al., 1989) was erroneously attributed to the kainate family, and thus reported multiple times for several years. It was later found to be an AMPA receptor subunit (**GluA1**). Three other AMPAR subunits (**GluA2-4**) were also found around the same time (Boulter et al., 1990; Keinänen et al., 1990; Nakanishi et al., 1990; Sakimura et al., 1990), which eventually completed the AMPAR subunit family we know today. The reason AMPARs were known as “quis” or quisqualate receptors before 1990 was that this was the first chemical substance used to stimulate the non-NMDAR component of the central EPSP. However, quisqualate also activated the kainate family of receptors, and as interest grew in the 1990s into dissecting the EPSP components, namely by separating the general “non-NMDAR” group, a more specific agonist had to be chosen, and this necessity fell on the **α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid** (AMPA) agonist, which had been synthesized ten years earlier (Krogsgaard-Larsen et al., 1980). Together with pharmacological antagonism of NMDARs with DL-2-amino-5-phosphonovalerate (APV), and of AMPA/kainate receptors with 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), the approximately dual nature of the central mammalian excitatory postsynaptic *current* (EPSC) was identified as being mostly composed of fast-AMPA and slow-NMDAR currents (Bashir et al., 1991; Spruston et al., 1995). Very interestingly, both excitatory synapses onto hippocampal pyramidal neurons (Hestrin et al., 1990) and inhibitory interneurons (Sah et al., 1990) showed these two components, which for historical interest we reproduce in **Figure 3.3.1**. Also, rise and decay times of each NMDAR and non-NMDAR (AMPA) components were comparable between

neuron types — however, the magnitude of the currents is much larger for excitatory synapses onto inhibitory interneurons than onto pyramidal cells. And there are other less obvious dissimilarities, such as in AMPAR composition, which is not the same in both pyramidal cells or inhibitory interneurons; indeed, the GluA2 subunit is notably absent from a great deal of inhibitory interneuron spine AMPARs, which is not the case with pyramidal neurons (Geiger et al., 1995; Leranath et al., 1996). AMPARs that don't have the GluA2 subunit are also calcium-permeable (Koh et al., 1995), and as such its currents have both a sodium- and calcium-component, as opposed to GluA2-containing AMPARs which carry only sodium currents (Spruston et al., 1995), in addition to potassium currents, which both have.

Meanwhile, overwhelming knowledge about the subunit variants, structure, auxiliary proteins, and function of ionotropic glutamate receptors, have accumulated over this three-decade period and is certainly outside the scope of this thesis, but very well reviewed

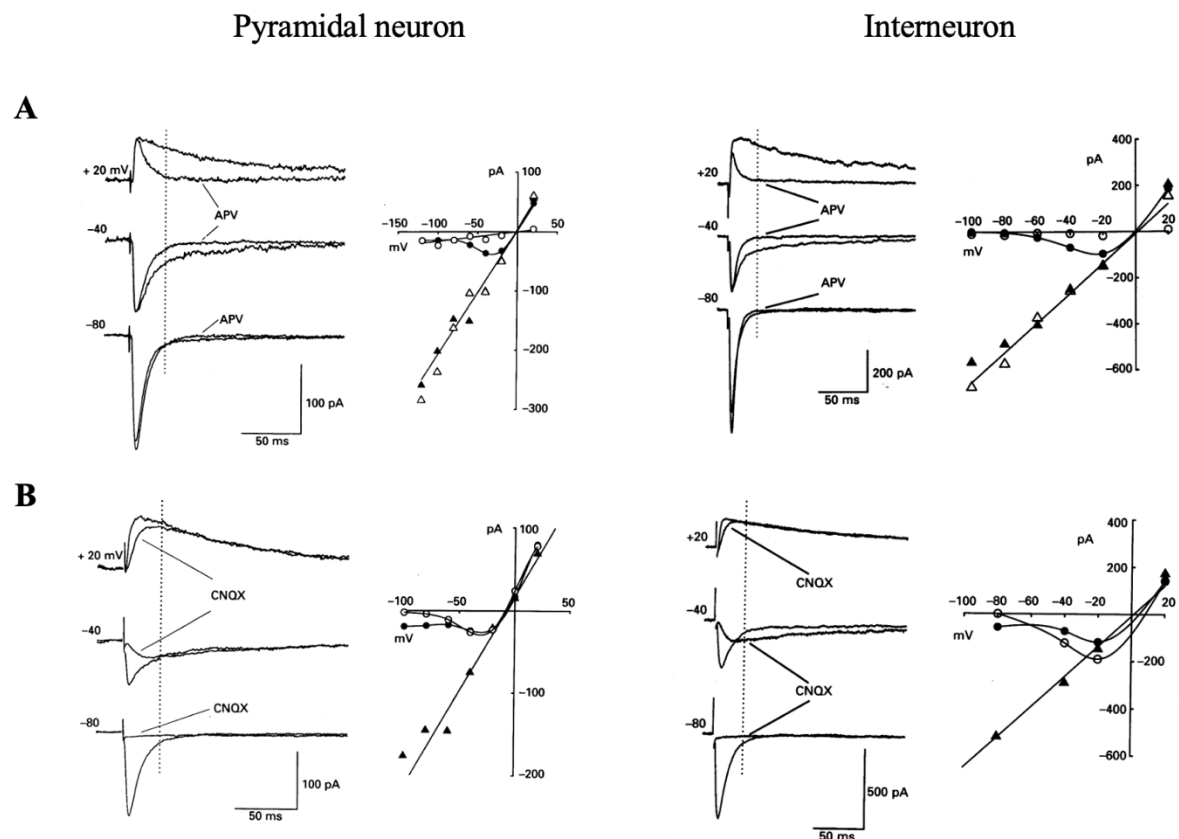


Figure 3.3.1. EPSC components historically recorded from CA1 pyramidal neurons (left) and interneurons (right) in the rat hippocampus. (A) Trace recordings at -80, -40 and +20 mV holding potentials with and without application of the NMDAR antagonist APV. Also shown are current-voltage relations at the trace peak without ($\blacktriangle$) and with ($\triangle$) APV, and 25 ms later (dotted line in the trace) without ($\bullet$) and with ($\circ$) APV. **(B)** Trace recordings at -80, -40 and +20 mV holding potentials with and without application of the AMPAR/kainate antagonist CNQX. Also shown are current-voltage relations at the trace peak without ($\blacktriangle$) CNQX, and 25 ms later (dotted line in the trace) without ($\bullet$) and with ($\circ$) CNQX. Left A and B from Hestrin et al. (1990); right A and B from Sah et al. (1990).

elsewhere (Hansen et al., 2021; Traynelis et al., 2010). **Figure 3.3.2** provides a general overview of the most important biochemical (and some biophysical) features of AMPARs. Like most secretory pathway proteins, AMPAR subunits are also subjected to several points of regulation, beginning with (a) **transcription** at the neuron nucleus to produce mRNAs (*transcription factor availability and binding*: e.g. SP4 and NRF-2 regulate GluA2 expression (Priya et al., 2014b, 2014a); *RNA editing*: e.g. Q/R-GluA2 exchange, rendering a channel bearing GluA2(R) essentially calcium-impermeable (Burnashev et al., 1992); *RNA alternative splicing*: e.g. the slow-desensitizing *flip* versus the fast-desensitizing *flop* variants of GluA2 (Koike et al., 2000)), (b) **mRNA levels** and **translation** in the cytoplasm (*nonsense-mediated decay (NMD)*: e.g. regulation of GluA1 surface levels by the UPF2 component (Notaras et al., 2020); *RNA silencing*: e.g. translational repression of GluA1 mRNA by miR-92a during homeostatic scaling (Letellier et al., 2014)), (c) **receptor assembly and stability** (*folding and tetramerization at the endoplasmic reticulum (ER)*: e.g. the crucial importance of cornichon homolog proteins (CNIHs) as ER cargo transporters, and in determining subunit composition of synaptic AMPA receptors (Certain et al., 2023; Herring et al., 2013)), (d) **transport to and from dendrites and spines** (*AMPA availability*: glutamate receptor interacting protein 1 (GRIP1) and Protein interacting with C kinase 1 (PICK1) have been suggested to work alongside to facilitate the delivery (H. Dong et al., 1997; Tan et al., 2020), or removal (Iwakura et al., 2001; Perez et al., 2001), respectively, of AMPARs from their functional areas; *intracellular transport*: both GluA1-interacting SAP97 and 4.1N proteins are fundamental for the control of the various phases of AMPAR expression and delivery (Bonnet et al., 2023)), and (e) **membrane stability** (*AMPA anchoring at the membrane*: many proteins, through their posttranslational modifications, directly or indirectly interact with AMPARs, or their auxiliary subunits, such as transmembrane AMPAR regulatory proteins (TARPs) to facilitate their stabilization close to, or at, the synaptic surface, e.g. the TARP stargazin/γ2, and the adaptors PSD-95, Homer1 and Shank (Dani et al., 2010; Hansen et al., 2021; Nair et al., 2013); *AMPA subunit degradation*: all receptor subunits undergo activity-dependent ubiquitination and ensuing proteasomal degradation (Widagdo et al., 2015)), all of which are affected through numerous environmental stimuli, and of course activity history of the synapses they are expressed in.

Whole-cell recordings at the *soma* and *apical* dendrites of hippocampal CA1 pyramidal neurons have confirmed that, as Rall predicted, the character of the EPSPs is not the same in both locations (**Figure 3.3.3**) (Magee & Cook, 2000). What the authors called *distance-dependent scaling* (but also referred to as *conductance* or *synaptic scaling*) was found to be somewhat related to increasing dendritic conductance in the soma-dendrite axis, using this model, and mainly due to progressively elevated numbers of AMPA receptors in that axis (Andrasfalvy & Magee, 2001; Smith et al., 2003) that seemed to correlate at least with

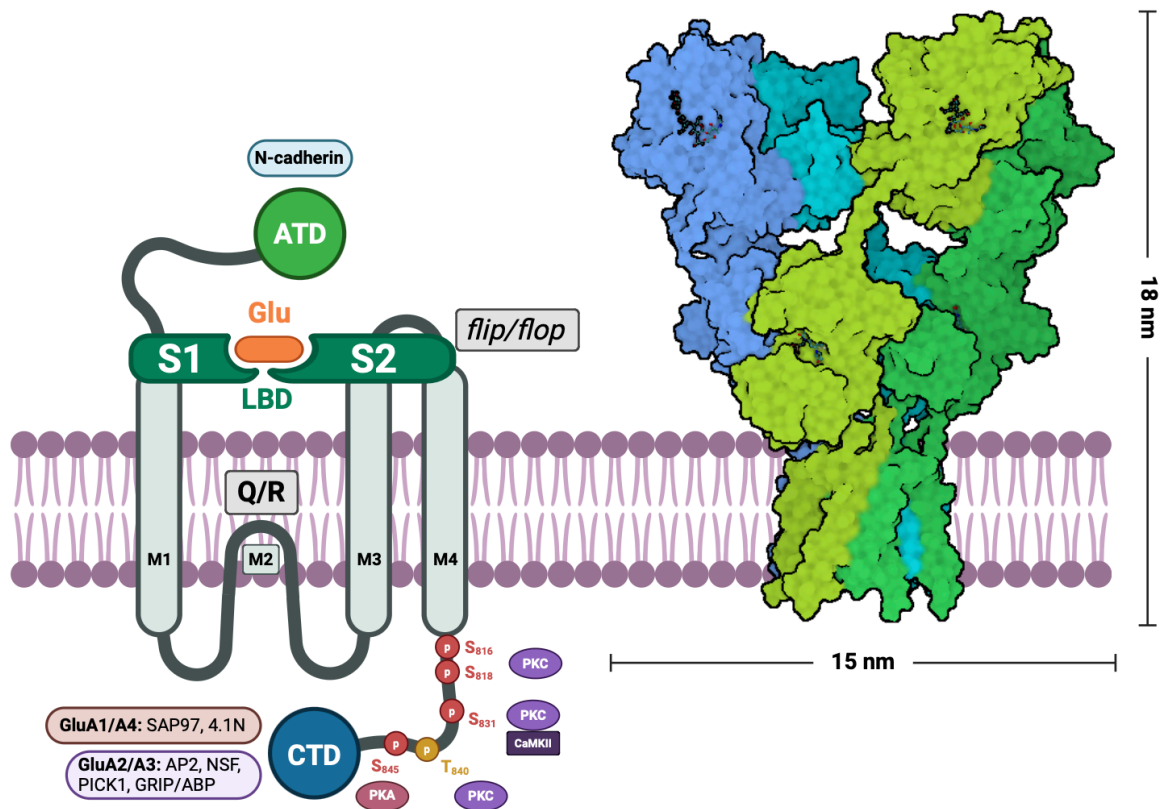


Figure 3.3.2. Summary of some important aspects of AMPAR biophysical and biochemical features.

Depicted to the right is PDB structure 3KG2, the AMPA-sensitive, homotetrameric, rat GluA2 receptor at 3.6 Å resolution (x-ray diffraction) in complex with a competitive antagonist (ZK 200775). On the left we present a rendering of a single, mostly generic subunit of the AMPAR channel. Indicated are the following domain features: amino-terminal domain (ATD), carboxyl-terminal domain (CTD), surface domains (S1/S2) forming the ligand-binding domain (LBD), and the four transmembrane domains (M1-M4). Along the polypeptide chain we included the Q/R RNA editing site and the *flip/flop* alternative splicing site of subunit GluA2, plus the most important phosphorylation sites at the CTD of the GluA1 subunit, along with their respective inducing kinases. Residues T₈₇₄ (Junk), Y₈₇₆ (src kinase) and S₈₈₀ (PKC) are also phosphorylated in the short-tail version of the GluA2 subunit, and the latter two seem to regulate the association of GluA2 with either GRIP/ABP or PICK1, but there is still some debate on this matter. Finally, subunit-specific binding partners at the CTD are shown, along with the interaction of the intercellular binding protein N-cadherin with the ATD of the AMPAR subunits.

the proportion of perforated synapses (Nicholson et al., 2006) and the expression of the accessory CNIH-2 protein (Shipman et al., 2013). This feature is not universal¹⁴, such that different types of neurons, or even the same type of neuron but from different local regions in the brain, may have different ways (including different AMPAR dependences) to process and convey information from distal dendrites to neuronal cell bodies. This realization is extremely important if one is to understand the role of AMPARs in *effective* neuronal communication within a circuit: their biophysical properties must be considered. Unfortunately,

¹⁴ Layer V pyramidal cortical neurons, for instance, do not show conductance scaling, as distal inputs to the dendritic tree attenuate more compared with more proximal inputs (Williams & Stuart, 2002).

working out the parameters affecting ion channel currents is not easy. Most channels can be mathematically modelled as total current functions of the single-channel currents (e.g. i_{AMPA}), number of channels (N) and the open probability (P_o) of a channel population. Given single-channel currents depend on both single-channel intrinsic conductance (γ_{AMPA}) and driving force ($V_m - E_{AMPA}$), AMPA receptor total currents can be modelled as $I_{AMPA} = \gamma_{AMPA}NP_o(V_m - E_{AMPA})$. Now, excitatory synapses start out silent, devoid of AMPARs, and are then very slowly populated by them (Friedman et al., 2000; Liao et al., 2001). The fast kinetics of AMPARs is mostly due to their sodium permeability (and to that of calcium, for GluA1 homotetramers), and a recent study has shown that after subthreshold electrical stimulation, the postsynaptic sodium contribution to the EPSP is almost entirely from AMPARs, with little contribution from either NMDARs or voltage-gated sodium channels (Miyazaki & Ross, 2017). Given stimulation removes a synapse from its silent state, in some conditions the number of AMPARs might be higher the more a synapse is stimulated. But how many AMPARs are there in a single spine? One of the first reports on this matter found ~300 receptors in membrane patches from CA1 pyramidal neurons (Spruston et al., 1995), but this involved an outside-out membrane patch, an area too extensive to correctly account for the number of receptors on a single spine. A subsequent, more spine-directed report found only

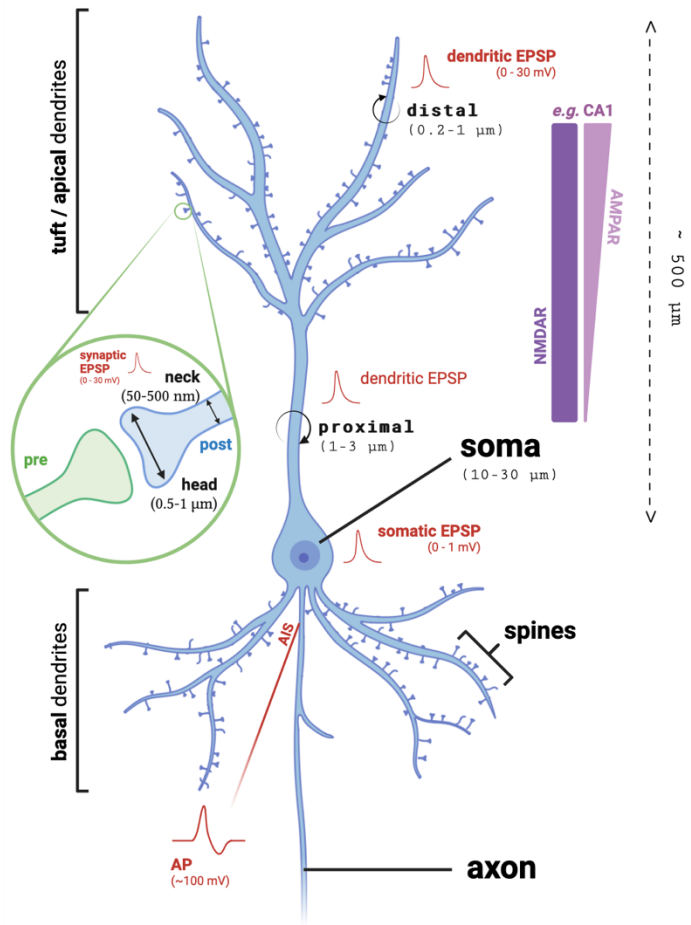


Figure 3.3.3. Overall scheme of a pyramidal neuron with a summary of some morphological and functional features. The pyramidal cell is typically composed of a cell body (soma) from which multiple dendritic branches, and an axon emerge. Dendrites form three identifiable regions: *basal*, usually to the side of the axonal initial segment (AIS), *proximal*, which may or not be branched, and a highly branched *apical* region forming a dendritic “tuft”. Although some aspiny neurons exist, the majority is covered with dendritic spines. Typical unitary EPSP ranges are indicated for synaptic/dendritic input (0-30 mV) and corresponding somatic recordings (< 1 mV), as well as the canonical action potential (AP) magnitude (~100 mV). Lengths in parenthesis represent average widths for the indicated structures. An example gradient of NMDA and AMPA glutamate receptors along the soma-dendritic axis is given for hippocampal CA1 pyramidal neurons.

between 46 and 147 (mean 82 ± 31) receptors per spine specifically in mushroom spines of the same neurons, with a maximal recorded current of ~ 19 pA (Matsuzaki et al., 2001), and an even more recent, detailed account using super-resolution imaging found AMPARs to be clustered in ~ 70 nm nanodomains in spines, ~ 27 receptors per cluster, and around 2.5 clusters per spine, resulting in ~ 68 AMPARs per spine, with a recorded current of 19.7 ± 1.3 pA (Nair et al., 2013), which is within the range of the Matsuzaki et al. study. The fact that there are ranges of receptor numbers makes sense if we think that at any point in time some spines have been more stimulated than others. These results are reassuring, but then again, there is a range of mushroom spine sizes (some even perforated) because of differential stimulation histories (Ofer et al., 2021; G. Stuart et al., 2016). AMPAR single channel conductances are also highly diverse and depend on both receptor subunit composition (e.g. homomeric GluA1 receptors show γ_{AMPA} values up to ~ 40 pS, higher than heteromeric GluA1/A2 receptors, with values of less than 10 pS), and the available glutamate concentration. Given receptors display multiple conductance sublevels as a function of LBD occupancy (Coombs & Cull-Candy, 2021), the more glutamate is available, the more LBDs in all four AMPAR subunits get filled in, and the higher the channel conductance. But given most AMPARs in the brain are tetramers of GluA1/A2 dimers¹⁵ (Schwenk et al., 2014), AMPAR overall conductance (and the most frequently found in the literature (Andrásfalvy & Magee, 2004; Coombs & Cull-Candy, 2021; Spruston et al., 1995; Vodyanoy et al., 1993)) is typically ~ 10 pS. We are thus able to use the experimentally available data to estimate the theoretical value of a single spine EPSP ($EPSP_{spine}$): the total current of a population of 68 channels, at the peak (we will assume most channels are open, $P_o \approx 1$) is around 19 pA, which means that single-channel currents are on the order of $i = I/(NP_o) = 19/(68 \times 1) \approx 0.28$ pA. Given $V_m - E_{AMPA} = i/\gamma_{AMPA}$, and we know $E_{AMPA} \approx 0$ mV, solving for the EPSP gives $V_m = 0.28/10 = 28$ mV. This estimate is remarkably in line with recent experimentally determined $EPSP_{spine}$ values of between 6.5 and 30.8 mV (from proximal/basal dendrites of L5 pyramidal neurons (Acker et al., 2016)), 26.70 ± 13.8 mV (from apical dendrites of L5 pyramidal neurons (Jayant et al., 2017)), between 4 and 27 mV (in cultured hippocampal neurons (Kwon et al., 2017)), 20.0 ± 9.5 mV (measured in pyramidal neurons from mouse somatosensory cortex L2/3 (Cornejo et al., 2021)), and between 8.4 ± 0.3 and 25 ± 3.4 mV (from basal dendrites of L2/3, L5, and L6 cortical pyramidal neurons (Weng et al., 2023)). Note that both the number of receptors per spine ($0 \sim 150$) and the single-channel conductance ($0 \sim 50$ pS) are allowed to vary if we fix the total current (~ 19 pA), and that these values may still be modulated by changes in the open probability of the population (which

¹⁵ And some GluA2/A3. Also, the cerebellum is the only known regional center where GluA4 has some importance, by substituting GluA1.

was here considered maximal, but may well not be), contributing to a dynamical adjustment of the observed 0 ~ 30 mV range (**Figure 3.3.3**). These spines were mostly stimulated by two-photon glutamate uncaging directly into a single spine, or via backpropagating action potentials (bAPs) generated at the soma or the parent dendrite, directed to a spine. All these studies also place the resulting somatic EPSP (EPSP_{soma}), once a single EPSP_{spine} travels down its parent dendrite, at around 0.2 – 0.8 mV, meaning the EPSP_{spine} is severely attenuated while moving towards the neuronal cell body¹⁶. This voltage filtering is, of course, beneficial, as otherwise 3-4 spine maximal signals would suffice to induce AP firing. It would be an excitatory mess.

So, in the old model, where only the somatic EPSP was considered, dendrites were simple information receptors, delivering incoming signals to the soma, mostly unaltered, where they were then linearly combined to provide an all-or-none decision to fire an AP at the AIS or not. In the modern model, however, each dendrite not only receives information from the exterior via its spines and synaptic contacts (for which AMPARs are most important), but also from other dendrites within the same tree, through either passive propagation or active propagation — dendritic spikes (dSpikes) — and even from the soma through bAPs, generating non-linear activity which may greatly influence somatic potentials to become non-linear summation devices (see Section 3.4). Increasing evidence indicates that a neuron's firing patterns, among other properties, are decided in dendritic trees, not the soma (Jia et al., 2010), and are hence probably responsible for the neural code.

AMPA composition, biochemistry and synaptic increases and decreases should not be considered separate from the neuronal types they are expressed in, nor apart from their dendritic properties. Furthermore, AMPARs are not the only players on the dendritic scene, and as research progresses so does our understanding of neuronal computations. As an example, the last two decades have rendered us a wealth of data on dendritic expression of several important classes of voltage-dependent channels, which contribute active conductances to the dendritic processes, and are ultimately responsible for directing the effect of EPSPs to the soma. At least four main types of voltage-gated channels have been found to regulate dendritic excitability: sodium (Na_v), calcium (Ca_v), and potassium (K_v) channels, as well as hyperpolarization-activated cation (HCN) channels (G. Stuart et al., 2016; G. J. Stuart & Spruston, 2015). These are differentially distributed across the dendritic plasma membranes of various neurons, and their integrative properties and firing behavior at central neurons vary greatly between different cell types (see Section 3.4). They contribute, for instance, to the properties of dSpikes following bAPs (Waters et al., 2005), but their

¹⁶ The mechanisms behind dendritic filtering are briefly explored in Section 3.4.

functions go way further, including computational response to spatial receptive fields (Moser et al., 2015), and the presence of both clustered and scattered spine modules among the dendrites of a single neuron responding to different features of the environment (Jia et al., 2010). Unfortunately, the hippocampus continues to be the most sought model, so that not much is known about the expression and distribution of these channels in amygdala neuronal dendrites.

Still, following AMPAR cloning, their mapping to many brain areas shortly began, implicating ample but differential distribution within neuronal cell bodies, dendrites and dendritic spines, e.g., in rat cerebral cortex, basal ganglia, limbic system, thalamus, cerebellum and brainstem (Martin et al., 1993). In the **amygdala** region, despite its abundant expression pattern, the same study found that the GluA1 subunit showed elevated immunoreactivity in both basolateral and posteromedial cortical nuclei, and that although the GluA2/3 subunit displayed similar patterns of expression in these areas, they were less intense (Martin et al., 1993). Somewhat differently, another study confirmed high levels of GluA1 in some, albeit few (7.5%) neurons of the basal amygdala nucleus resembling previously identified non-pyramidal neurons, but found high levels of GluA2/3 in almost 91% of the pyramidal-like neurons analyzed in this nucleus, and little or no reactivity in non-pyramidal cells (McDonald, 1994). Finding general immunoreactivity in particular areas might be interesting if the area is homogeneous for a certain neuron type population (such as is the case of the striatum, where almost all neurons are GABAergic), but this is not so if populations are heterogeneous, given selected groups of neurons may then display different functional assemblies of receptor subunits. At an experimental level, both the amygdala nuclei and their neurons are not easily discernible without proper staining of selective markers, morphological characterization in relation to other more easily identifiable structures, or careful assessment of electrophysiological properties. And indeed, for instance, the **basolateral (BL) nucleus** has a highly heterogeneous collection of excitatory neurons that has been shown to spatially covary in molecular, cellular, and circuit properties (O’Leary et al., 2020). Similarly, the **central (Ce) nucleus** is mostly composed of striatal-like medium spiny neurons, which are almost all GABAergic, but which also display an heterogeneous population of inhibitory interneurons (J.-N. Li et al., 2022). Given AMPARs are expressed at the spines of both excitatory and inhibitory neurons (inputted by glutamatergic neurons), discerning receptor subunit distribution within these neuronal types would importantly add to their functional significance there. Unfortunately, as we mentioned before, very few studies have addressed the precise location of AMPAR subunit expression in the BL (the subject of the present thesis) or within BL neurons, and the ones that have are either somewhat imprecise or contradict each other. The majority of neurons in the BL are excitatory, pyramidal-like cells, with reported populations e.g. at 85% by morphological tracing (McDonald, 1992),

93% via morphological staining and intracellular recordings (Washburn & Moises, 1992), and again 93% in an independent study using BL whole-cell recordings (Mahanty & Sah, 1998), with the remaining few 7-15% within all of these reports displaying inhibitory interneuron-like characteristics. Some early studies confirmed that both pyramidal-like cells and inhibitory interneurons in the BL express the GluA1 subunit (although not agreeing on how strongly), but that while pyramidal-like neurons exhibit elevated GluA2 expression, non-pyramidal cells (mostly inhibitory interneurons) have very low, if any, GluA2 immunoreactivity (Farb et al., 1995; McDonald, 1994, 1996), which would account for their high calcium-permeability and AMPAR inward rectification¹⁷ (Mahanty & Sah, 1998). Few of these studies explored subcellular details. One of them found immunoreactivity for both GluA1 and GluA2/3 in neuronal somata, large and small dendritic shafts, and dendritic spines, seldomly confined to the postsynaptic density (PSD) (Farb et al., 1995). Given their expression at PSDs, these neurons are probably of the excitatory, pyramidal type. A few years later, a different study criticizing the use of polyclonal antiserum in previous research for being unable to discern between GluA2 and GluA3 subunits, used an anti-GluA2 monoclonal antibody that had been shown specific for this AMPAR subunit. The results revealed extensive, uniform GluA2 immunoreactivity throughout the rat BL, subcellularly in neuronal perikarya and proximal dendrites, but that was equally present in both morphological pyramidal-like and non-pyramidal cells (He et al., 1999). The latter were confirmed as GABAergic and the vast majority (~94%) colocalized with GluA2. Plus, electron microscopy with peroxidase pre-embedding again showed GluA2 subunits in both non-GABAergic (pyramidal) and GABAergic (non-pyramidal) neurons. This seemed to be at odds with previous work on BL AMPARs. But using the immunogold technique, the authors somewhat reconciled with the literature by finding that, besides functionally significant GluA2 labelling being confined to asymmetric (type 1) synapses to both GABAergic and non-GABAergic spines and dendritic shafts, the number of GluA2 immunogold particles was actually ~69% higher in the latter (He et al., 1999). This is in line with electrophysiological recordings showing inward rectification of AMPARs specifically in BL inhibitory interneurons (Rainnie et al., 1993; Washburn & Moises, 1992).

The controversy surrounding the expression of GluA2 in the rat amygdala BL nucleus was revisited one final time up to this day, twenty years ago, again using a GluA2 antibody

¹⁷ Rectification is a property of single ion channel I-V relationships in which the channel displays non-linear current-voltage dependence. Following accepted convention, given calcium-permeable AMPARs show limited current above 0 mV, their preferential current-pass is negative, and hence *inward*, meaning they only allow sodium and calcium ions in while the membrane voltage is negative (which, truth be told, is the situation for most synapses).

that also recognizes the GluA3 subunit, plus whole-cell voltage clamp recordings from BL neurons. The researchers accordingly found a large majority of BL neurons to be GluA2⁺ in both perikarya and proximal dendrites under the light microscope. A closer look via electron microscopy confirmed a preponderance of synapses onto spines/PSDs (86%), relative to dendritic shafts (14%). Still, of all BL synapses studied, only 11.5% were GluA2⁺, keeping in with spine (77%) and shaft (23%) distributions. Concurrently, whole-cell voltage-clamp recordings showed 72% of the 17 chosen BL neurons exhibited inwardly rectifying AMPAR synaptic currents (Gryder et al., 2005). Naturally, the major caveat of this study is the lack of segregation between pyramidal-like and inhibitory interneuron cell types. Many of the observed neurons are presumed non-pyramidal but even the n = 17 population size used for voltage-clamp recordings is too low to draw sound conclusions. A slightly more recent account labelling receptors via immunoelectron microscopy, found that a high percentage of thalamo-La spines contained both GluA2 (80%) and GluA3 (83%), but given the authors have not distinguished between cells types, this result bears little functional significance

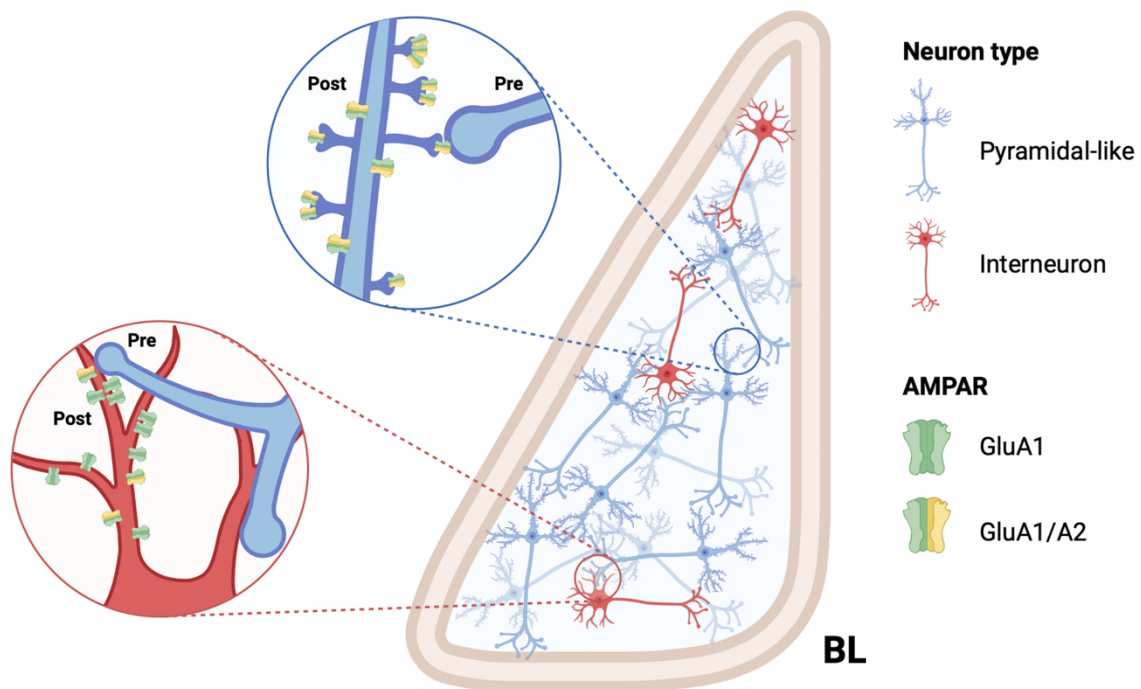


Figure 3.3.4. Summary of AMPAR subunit expression in the amygdala BL neurons. According to the available literature, the vast majority of BL neurons are pyramidal-like, glutamatergic cells (~90%), the remaining being GABAergic interneurons (~10%). GluA2-containing AMPARs are found in dendrites of both cell types, and they colocalize with GABA in > 90% of interneurons; still, most of this superposition occurs in perikaryal and dendritic shafts. In excitatory synapses to either neuron type, most GluA1/A2 receptors are found in dendritic spines (~80%) and proximal dendritic shafts (~20%), but they are far more abundant in synapses to non-GABAergic cells. As a result, interneurons have many synapses with GluA1-only AMPARs, rendering the receptors calcium-permeable and inwardly rectifying, as frequently appreciated in electrophysiological recordings.

(Radley et al., 2007). So far, the He et al. (1999) study still provides the most complete and compelling data to date regarding the distribution of AMPAR subunits in the amygdala BL nucleus (He et al., 1999) (**Figure 3.3.4**). Every study presented above was conducted using male Sprague-Dawley rats, the same animal strain we used in all experiments explored in the present thesis (Moraes & Hardt, 2023). Still, the main conclusions are similar for other strains: for instance, one study using another albino rat (Wistar) found the same pattern of AMPAR subunit segregation between amygdala pyramidal and non-pyramidal cells in the La nucleus (Sah & Lopez De Armentia, 2003). Nevertheless, knowledge about cortical and hippocampal dendritic distributions of AMPARs seems overwhelming when compared with what we know on BL neurons. Clearly, much work on this theme lies ahead.

Figure 3.3.4 reminds us that in a small amount of tissue collected from the amygdala there will be a mix of excitatory and inhibitory neurons, along with astrocytes, oligodendrocytes, and extracellular matrices, and many other proteins. Structures receiving many inputs, such as the amygdala, are at the center of microcircuitry crossroads, and how their cellular and molecular modifications relate to behavioral change is highly complex. Attempts to parallel molecular and behavioral systems data have theoretically divided the creation of a “synaptic trace” into several stages (generation, stabilization, consolidation, and maintenance) akin to those for “memory systems’ traces” (formation, consolidation, and maintenance/forgetting). But given neural processes, as many other biological entities, are largely forever dynamic, in the simple sense of continuously changing over time, which alludes to Francis Crick’s “memory and molecular turnover” paradox (Crick, 1984) (we have seen this for spine pruning in Section 3.2), there is no true beginning nor end to any of them: many rounds of consolidation adjustments may never represent any truly *new* trace generation, only recycling of whatever *was*. Take the so-called “up states”, which refer to spontaneous NMDAR-dependent calcium waves invading neuronal spines, thought to be part of trace consolidation processes. Indeed, *in vivo*, at any given up state, a single auditory cortical neuron’s dendritic tree will spontaneously activate about 500 non-clustered spines (Chen et al., 2013), the same set that is active during sensory stimulation. This is a non-trivial issue: it means there are many shades to traces or “memories”, and that activation patterns recur in many forms and are able to change over time. Naturally, recall vividness is impossible to assess in animal models. That is why, as we have explored in our paper (Moraes & Hardt, 2023), a population of animals may display a range of behaviors following exposure to a very standard, and rigorously applied auditory fear conditioning (AFC) protocol: our data provided a wide range of different percent values of fear generalization during test Pre-CS, ranging from zero to 100%, and quite a wide range of freezing responses to CS presentation. Encoding and expression is not the same for all individuals, despite their similar

early-life experiences, experimental manipulations, and even shared circuitry conducting to freezing behavior (**Figure 3.3.5**).

As we have seen, amygdala BL neurons may subcellularly express both AMPAR GluA1 and GluA2 subunits. This structure, or at least the La division, has been the subject of intense research for several decades regarding its participation in the acquisition and retrieval of several forms of **fear memory**, an important topic to the present thesis. The LTP phenomenon has also been studied in the La nucleus in the context of auditory fear condition-

ing¹⁸: when auditory tone-evoked responses are re-probed following conditioning, they show changes similar to those obtained after LTP induction (Rogan et al., 1997), further hinting on LTP as an excitatory means for trace preservation. However, the first study directly showing the involvement of AMPARs in cortical and thalamic inputs to the amygdala, which are related to fear memory acquisition, came only six years following the cloning of these receptors (X. F. Li et al., 1996). Two years later another group confirmed that AMPARs mediate glutamatergic transmission to inhibitory interneurons in the BLa nucleus, and that these associated with amplitude increases in GABA_A transmission from these neurons onto amygdala pyramidal-like cells (Mahanty & Sah, 1998). They later pro-

vided more evidence for the difference in spine synaptic AMPAR subunit compositions (GluA1 vs GluA2) between both types of neurons (pyramidal vs inhibitory interneuron), as we discussed above (Sah & Lopez De Armentia, 2003). Thus, by the early 21st century, a

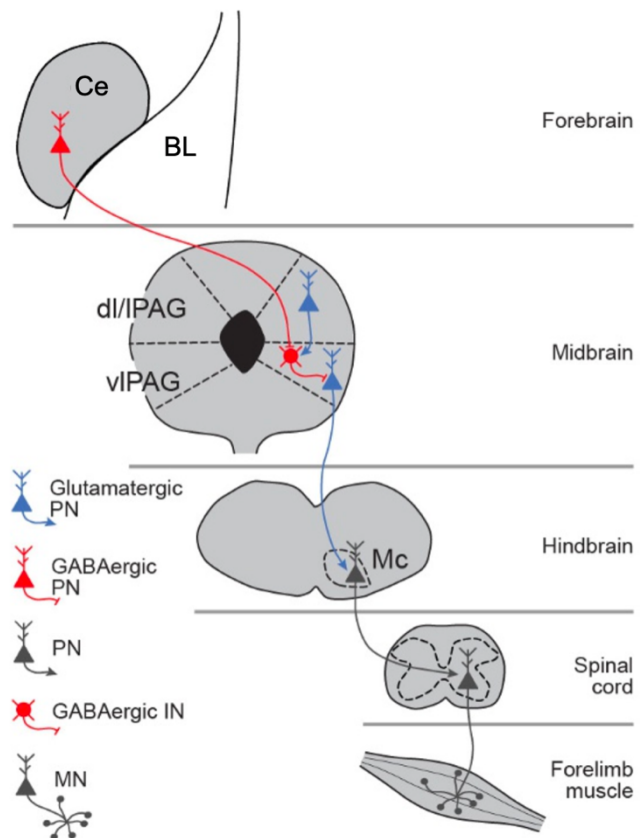


Figure 3.3.5. Summary of mouse cells and circuits involved in freezing behavior production. Ce, central nucleus; BL, basolateral nucleus; dl/IPAG, dorsolateral / lateral periaqueductal grey (PAG); vlPAG, ventrolateral PAG; PN, projection neuron; Mc, magnocellular nucleus; IN, interneuron; MN, motor neuron. Adapted from Tovote et al. (2016).

¹⁸ Amygdala expression and changes in AMPAR subunit content have also been explored using the fear-potentiated startle (FPS) paradigm, but we will not refer these studies in the present work.

picture was slowly emerging in which at least some excitatory inputs to the B(La) nuclei were onto inhibitory interneurons expressing GluA2-lacking AMPARs, and with outputs to downstream pyramidal neurons, leading to their inhibition via GABAergic transmission (Wiltgen et al., 2009). This was further shown to occur during auditory fear memory acquisition (Szinyei et al., 2007), and brought support to a complicated interplay between excitation and inhibition in the amygdala assisting fear encoding.

At these early times, the group of Patricia Shinnick-Gallagher sought a presynaptic role for AMPAR involvement in BL encoding, most frequently using pair-pulse facilitation or depression (McKernan & Shinnick-Gallagher, 1997; Zinebi et al., 2001, 2002), but at the end, not disregarding a presynaptic contribution ((Choi et al., 2021)), most studies came to favor the postsynaptic view, which is still accepted today and involves insertion and removal of AMPARs from spine PSDs (Yu et al., 2008). As research continued, a 2005 seminal study elegantly revealed the involvement of GluA1 expression in the La nucleus during LTP development at thalamo-La synapses, and offered a behavioral demonstration of related fear acquisition and retrieval, where researchers used auditory fear conditioning to show that if as few as 10-20% of La neurons were impaired for GluA1 insertion, the efficiency of expression of the fear memory would be visibly reduced (Rumpel et al., 2005). Similarly to AFC, visual-cue (light) fear conditioning applied to male Sprague-Dawley rats confirmed that, following this type of conditioning, the content of GluA1 also increased substantially in both biotinylated slices and synaptosomes¹⁹ isolated from the BL nuclei over the next 24 h (Yeh et al., 2006). Yeh et al. also observed that tetanic stimulation of external capsule afferents to the La nucleus generated LTP accompanied by an increase in GluA1, at least up to 6 h following HFS. Quite interestingly, the same group later confirmed that a visual (light) fear extinction protocol²⁰ applied within 1 h of conditioning (but not 24 h after), or low-frequency stimulation (LFS) under an NMDAR partial glycine site agonist, within 1 h of HFS, both reduced the GluA1 content in the same amygdala structures (Mao et al., 2006). Further, complete abrogation of GluA1 using a KO mouse model, rendered these animals

¹⁹ Synaptosomes, also called *synaptoneuroosomes*, are the result of neural tissue homogenization and fractionation, whereby spines involved in synaptic contacts are broken below the neck and resealed. These preparations may contain presynaptic material, as well as the PSDs and adjacent extra-synaptic membranes.

²⁰ Fear extinction is an experimental protocol whereby a fear-conditioned subject animal is repeatedly exposed to the CS alone in a safe context, different from the conditioning one, which usually leads to a decrease in the presentation of the conditioned response (e.g. motor freezing). The phenomenon of *spontaneous recovery* of the CS-US association, hence of the conditioned response, is frequently seen if the CS is again presented in the safe context some time after the extinction protocol is stopped. These observations have hinted on the idea that fear extinction is not erasure but new learning, which inhibits the CS-US association.

uncapable of learning either contextual or auditory fear conditioning, and impaired generation of thalamo-amygdala LTP (Humeau et al., 2007), which does not happen if the GluA1 loss is only partial (Feyder et al., 2007) (this is at odds with the Rumpel et al. (2005) study, probably due to developmental compensation typically observed in KO strains).

For about a decade since the emergence of interest on the role of AMPARs in fear conditioning learning and expression, experiments were mostly concentrated on the GluA1 subunit which, as mentioned above, is the most ubiquitous isoform. Indeed, contrary to the GluA2 subunit, which is almost absent from amygdala non-pyramidal neurons, GluA1 is present in spines of both excitatory and inhibitory amygdala neuron dendrites. But eventually attention started to be given to the GluA2 subunit as well. In 2007, the group of Sukwoo Choi further hinted at a link between fear extinction and the removal of, this time, the GluA2 subunit from La synapses by showing that inhibiting this subunit's endocytosis prevented LTP depotentiation and attenuated fear extinction (J. Kim et al., 2007). It seems the atypical protein kinase C isoform, protein kinase M ζ (PKM ζ), is involved in keeping GluA2 at synaptic PSDs by some as yet unknown mechanism, such that inhibiting PKM ζ impairs auditory and contextual fear memory recall, but blocking the removal of GluA2 from spines prevents this effect (Migues et al., 2010; Serrano et al., 2008). Although disrupting the interaction of the GluA2 subunit with the N-ethylmaleimide-sensitive factor (NSF) (see **Figure 3.3.2**) in the La nucleus also impairs conditioning via auditory fear (Joels & Lamprecht, 2010), these studies still seem to assign GluA2 a more important function in auditory fear maintenance (supported by PKM ζ) than in its formation, given GluA1 is readily increased in both spines (99%) and shafts (167%) of La nuclei up to 40 min following conditioning, but GluA2 keeps about the same levels during that period (Nedelescu et al., 2010), and only seems to change *after*.

The study of the importance of AMPAR GluA1 and GluA2 subunit functional expression in several amygdala nuclei, in the context of AFC (or other forms of fear conditioning), hasn't advanced substantially during the last decade. The group of Sukwoo Choi published a paper in 2013 indicating that upon AFC memory retrieval, La synapses exchange GluA2-containing for GluA2-lacking AMPARs, and then insert them back again during synaptic reconsolidation²¹ (Hong et al., 2013), but no distinction between cell types was made. Since then, some interest grew on the role of AMPAR subunit **posttranslational modifications (PTMs)** instead, as some of these had been already shown to affect several aspects of AMPAR trafficking and function in many *in vitro* preparations. For instance, GluA1 S845A knock-in

²¹ Reconsolidation is a hypothetical process of synaptic rearrangement and re-stabilization following reactivation, or so-called updating, of a memory.

rats (but not S831A mutants) failed to exchange GluA2-containing for GluA2-lacking AMPARs in thalamo-La synapses following AFC (i.e., during trace generation and stabilization), but these synapses still got potentiated and animals were capable of learning the CS-US association, and even of extinction learning (Clem & Huganir, 2010). Given compensatory mechanisms can mask the functional importance of GluA1 S845 phosphorylation (which is a PKA target), another group looked at the protein subunit itself by probing native pS845 levels following one retrieval event (24 h after conditioning) or a second retrieval event (1 h after the first) subsequently to AFC (Jarome et al., 2012). They found that while one retrieval event produces a significant increase in GluA1 pS845 levels within 1 h, the second returns phosphorylation levels to a basal state. However, if the spacing between retrieval events increased from 1 h to 24 h, then the second retrieval event retained an increased pS845 level. These fluctuations were found for GluA1 phosphorylation only, as total GluA1 was reported not to change; however, this might be because the authors evaluated protein levels in synaptosomes, which make no distinction between synaptic and extra-synaptic membranes. Still, given pS845 is linked to AMPAR trafficking events, a specific increase in total synaptic GluA1, e.g. via *de novo* transcription and translation, cannot be ruled out. Indeed, the activation of the transcription factor cAMP response-element binding protein (CREB) upon memory retrieval both correlates with fear memory lability and GluA1-pS845 levels (J. Kim et al., 2014), which is in line with both being substrates to PKA. Another group found that the number of CS-US pairings during AFC determined whether the association was more or less prone to destabilize upon retrieval, and that a higher number of CS-US pairings led to an increased BL GluN2A/GluN2B ratio, relative to when fewer pairings were delivered, with a corresponding decline in GluA1-pS845 (Holehonnur et al., 2016), which is in line with experiments in which reconsolidation is blocked. Thus, these authors proposed that phosphorylation of GluA1's C-terminal at S845 following fear recall is necessary for memory updating. This is an important observation, considering the various AFC protocols used in the literature: in order to link AMPAR metabolism to fear memory expression, it is important to consider whether results stem from studies with many or few CS-US pairings, as these induce dissimilar biochemical outcomes. The proper understanding of these will then guide the molecular logic behind further behavioral manipulations. For instance, the Sukwoo Choi group found that following extinction of AFC, the CS-US association renewed²² if the renewal was either of the ABA or ABC type, in a way that was dependent on

²² Following AFC extinction, usually achieved in a context different from the AFC context, three general relapse phenomena have been studied: *reinstatement* involves simple presentation of the US (foot shock); *spontaneous recovery* involves the passage of time (hence, a form of forgetting of the extinction learning); *fear renewal* occurs

GluA1 phosphorylation of S831 (a CaMKII and PKC target) in the amygdala La nucleus of male Sprague-Dawley rats (Lee et al., 2013; K. Park et al., 2014). Previous research had shown that extinction of the CS-US association somewhat involved a decrement in La GluA1 and GluA2 contents (see above), complicating matters. But since pS831 enhances AMPAR single-channel conductance, the authors suggested it could play a role in renewing signal spreading within parts of the original AFC memory neuronal substrate, despite some GluA1 loss, thus helping inhibit extinction learning. Still of note, immediate fear extinction, in which an extinction session is applied within 30 min of AFC, failed to produce stable CS-US extinction learning, and this was shown to involve calcineurin-mediated dephosphorylation of GluA1-pS845, which reduced its levels in BL total protein samples (Hollis et al., 2016). Albeit an interesting result, it is hard to reconcile with our previous discussion given so many things are happening in BL neurons at this time point following AFC, and the authors did not discriminate between extra-synaptic and synaptic sites, much less different neurons in the BL. Unfortunately, in the last decade, not much has been published that furthers our knowledge of these processes in the AFC-BL axis (**Figure 3.3.6**). The only exploration of GluA2 involvement in PTMs was by the group of Fred Helmstetter, that showed that upon fear memory retrieval and update, if GluA2 endocytosis was blocked in the amygdala, Lysine-48 ubiquitination previously found on several substrates (Jarome et al., 2011) was reduced (Ferrara et al., 2019), but it is difficult to envision a direct link between an ionotropic receptor and the ubiquitin-proteasome system (UPS), such that these observations are probably concomitant, but not consequential. Still, they confirmed GluA2 endocytosis following fear memory recall, which is in line with previous studies showing increased rectification in amygdala neurons. Furthermore, Choi et al. (2021) found that at least part of La synaptic input to Ba nuclei displayed presynaptic LTP following auditory fear conditioning, involving increased neurotransmitter release but apparently no postsynaptic AMPAR changes there (Choi et al., 2021), reviving some interest in presynaptic memory mechanisms.

Several other biological players have been demonstrated to influence AMPAR dynamics within the AFC-BL axis, but many are either isolated experiments or provide poorly demonstrated AMPAR/fear relationships. I will thus only refer the involvement of NMDARs. While they certainly have an important role in fear memory acquisition, expression, and extinction (Zimmerman & Maren, 2010) alongside AMPARs, there is still some uncertainty as to which extent receptor differential composition, including the presence or absence of the GluN2B

when the animal is presented the CS alone (tone) in the original AFC chamber (ABA renewal) or in a different chamber (ABC renewal).

subunit in neurons, is involved in fear conditioning encoding. While some initial data indicated GluN2B is expressed in both GABA⁺ and GABA⁻ La nuclei neurons receiving inputs from both thalamic and cortical sources (Szinyei et al., 2003), others have later found that La nucleus' inhibitory interneurons (GABA⁺) directly involved in LTP formation there (hence, potentially linked to fear memory processing) *lack* expression of GluN2B and receive mostly inputs from the cortex (Polepalli et al., 2010). This is not to say that NMDARs are not contributing, but that this particular subunit, which has received much attention over the years, is possibly not as much involved in fear plasticity at La inhibitory interneurons. Still, many

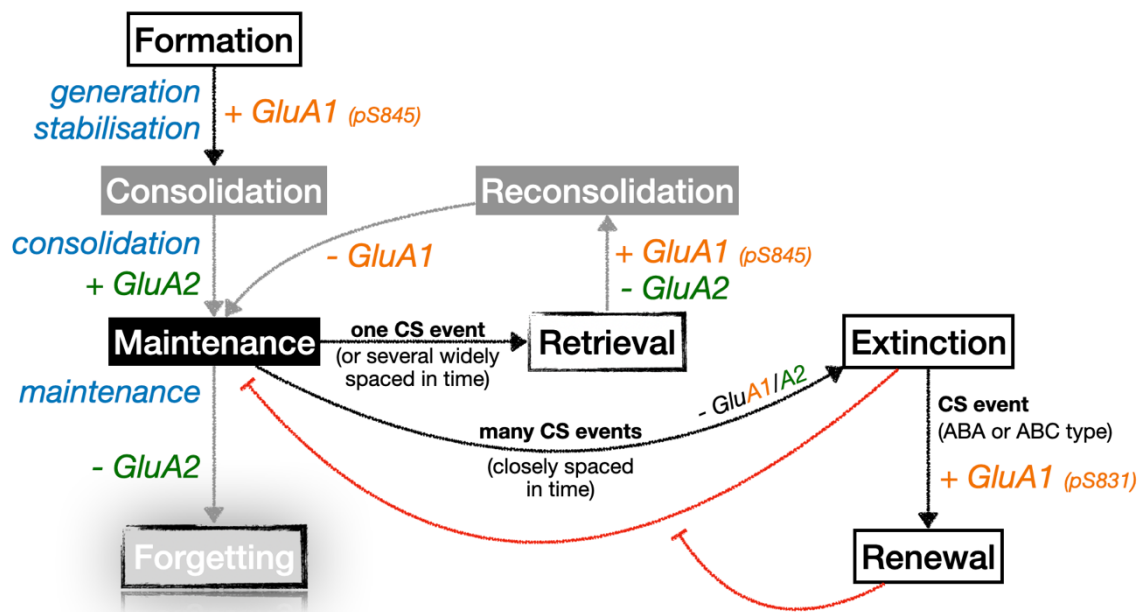


Figure 3.3.6. General summary of the categorization of fear memory processes described in the literature following AFC, involving the B(L) nuclei of the amygdala and expression of AMPARs. Cued-fear associations during fear memory formation start by generating and stabilizing a new trace (in blue), which has been directly correlated to LTP development, with GluA1 phosphorylation at S845 and synaptic insertion in BL neurons. The consolidation of the CS-US association follows consolidation of the memory trace by increased insertion of GluA2-containing AMPARs, which help maintain the memory stable. If a single retrieval event, or a widely spaced series of them, occurs, GluA1 is again phosphorylated at S845 and delivered to synaptic sites, while GluA2 is removed. The inverse ensues if the memory is allowed to reconsolidate back to the maintenance state. However, if many retrieval events, closely spaced in time, are presented to the animal, no S845 phosphorylation of GluA1 occurs, and the CS-US association is weakened (extinguished) with concomitant removal of AMPARs from some yet unknown BL cells. Renewal of the fear memory can occur if the CS is presented in the original conditioning context (ABA), or in a new context (ABC), which is thought to inhibit extinction learning in a way that is mediated by increased phosphorylation of GluA1 at S831, with a resulting increase in single-channel conductance. Finally, it is not clear if GluA2 removal is linked to memory forgetting, or if memories are indeed ever totally forgotten, but given inhibition of PKM ζ removes GluA2 from BL synapses and renders animals incapable of recalling the CS-US association, it is tempting to suggest (and supported by research in other brain areas) that removal of GluA2 is part of a forgetting process.

studies have found that the GluN2B subunit is important for fear extinction and depotentiation of conditioning-induced thalamic-La inputs (S. Park et al., 2012), but as cell type was not discriminated, this influence may involve La pyramidal-like neurons instead. Glutamate receptors are ligand-gated ion channels, so that their main function is to provide a flux of ions through somatic, and dendritic shaft and spine membranes, and thus help produce relevant EPSP codes. NMDARs are invariably linked to calcium influx (though they are also permeable to sodium and potassium), and its concentration controls a plethora of biochemical checkpoints, including AMPAR trafficking and single-channel conductance properties. Indeed, calcium metabolism is so fundamental that if one knocks out the L-type voltage-gated $\text{Ca}_v1.2$ channel in thalamo-amygdala synapses, it not only impairs the acquisition of conditioned fear, but cells unfruitfully try to compensate this loss by over-expressing GluA2-lacking AMPARs which, as mentioned above, also mediate calcium entry (Langwieser et al., 2010). Interestingly, while NMDARs in the basolateral amygdala (BL) are clearly necessary for forming initial CS–US associations during fear memory acquisition, they may not be required when a second conditioned stimulus (CS2) is paired with a US that was already associated with a first CS (CS1). In this case, learning proceeds without BL NMDARs but instead depends on GluA2-lacking AMPARs in BL inhibitory interneurons (Williams-Spooner et al., 2022). This suggests that NMDAR involvement may depend on how predictable the stimulus is.

The literature is still very poor on the involvement of AMPAR expression at specific amygdala nuclei synapses in the development of fear memory **generalization**, an issue we have recently tackled in the context of the present thesis (Moraes & Hardt, 2023). Fear generalization is at the heart of important human conditions such as posttraumatic stress disorder (PTSD), in which the patient is no longer capable of properly discriminating between safe and aversive signals from its surroundings. However, other than these extreme, pathological cases, we and others have found considerable variation in the individual fear discriminatory power within a population of animals (Duvarci et al., 2009). This indicates that there are biological differences, be it in biochemical pathways or within interregional center's microcircuitry in the brain, supporting this variation in behavioral responses. But the fact that most of the literature is built upon population *averages* only allows for major population shifts to be valued. As discussed before, the general increase in the expression of the GluA1 subunit following fear conditioning protocols has been established around two decades ago (Rumpel et al., 2005; Yeh et al., 2006). But as we have been alerting to, it is, however, important to know *where* in the circuit, or at least among the several amygdala nuclei, is the expression of this subunit modulated. Also, it is important to consider the type of stimulus, considering the brain does not deal with all environmental signals in the same manner. Of the several known amygdala nuclei, damage to the La and Ce nuclei interferes with fear

encoding following AFC (Nader et al., 2001), while lesioning the basal (Ba) nucleus is essential to disrupt *contextual* fear conditioning (CFC) (Goosens & Maren, 2001; Onishi & Xavier, 2010), when the pairing with the aversive stimulus is not with a particular cue (such as the auditory cue) but with those cues that together constitute the surroundings (the context). This distinction has found multiple reflection in experiments probing the involvement of other structures providing input to the amygdala. For example, the bed nucleus of the stria terminalis (BNST) has been shown to be critical for the expression of contextual fear, but not for fear to an auditory stimulus (Zimmerman & Maren, 2011). Indeed, BNST-amygdala interactions determine a great deal of inter-individual variations found in fear generalization and anxiety rat phenotypes (Duvarci et al., 2009). The BNST is just an example, but there is contribution from other relevant brain areas, such as the cortex, to amygdala-related fear generalization. Our concern in this section is with the involvement of AMPARs and, as it happens, besides our 2023 paper, there are only two other recent (2022) publications touching this issue. In one of them, the authors used a GluA1-C811S mutant mouse, which cannot undergo S-palmytoilation of its GluA1 subunits (Oota-Ishigaki et al., 2022). This interferes with AMPAR trafficking, given GluA1 palmytoilation inhibits the interaction between this subunit and protein 4.1N, known to drive AMPAR insertion into synapses (T. Hayashi et al., 2005). GluA1-C811S mice display enhanced seizure susceptibility and robust LTP-induced spine enlargement, accompanied by synaptic PSD increase of GluA1 levels in the amygdala. When these animals underwent AFC, CFC and extinction training, although remote fear expression following AFC was not much different compared to wild-type animals, the mutant mice strongly generalized the CFC memory over time and it was difficult to extinguish it. It is not clear if during AFC the animals were allowed to explore the conditioning box before conditioning or if they were placed there and immediately presented with the CS-US protocol (Oota-Ishigaki et al., 2022). Since our experimental design included a 2-minute exploration period prior to auditory fear conditioning (AFC) (Moraes & Hardt, 2023), and our observed fear generalization occurred during the Pre-CS phase of testing, we cannot rule out the possibility that contextual encoding contributed to this effect. Thus, context may play a role in the fear generalization we observed. The paper by Oota-Ishigaki et al. therefore hints at possible differences in GluA1 S-palmytoilation levels between individuals displaying dissimilar susceptibility to generalize fear, with less S-palmytoilation correlating with higher PSD GluA1 levels, and elevated fear generalization. The second paper (Hoffman et al., 2022), explored how traumatic brain injury (TBI) affects fear learning. Of interest in the present context, they found that individual total BL GluA1 levels (but not GluA2) positively correlated with the level of tone fear generalization (Hoffman et al., 2022), which is a finding echoing ours, although we did not use a TBI model. Whether the injury itself caused this relationship is not clear, as it was not reported if the correlation data was

only for injured animals, or if it included all data from both injured and sham conditions. Given this was a population effect, it is possible the correlation is a natural phenomenon, as we have observed, but this possibility can only be resolved by further experimentation. Research into the involvement of AMPAR metabolism in fear generalization mediated by the amygdala is still at its infancy but is nevertheless a promising relationship to keep in mind, as much is yet to be investigated on this matter.

It is increasingly becoming insufficient to affirm that changes in synaptic AMPAR levels on a particular brain area cause a particular memory or behavior, without referring to the cell type in which this happens (and this, I acknowledge, is a major caveat of my own research, and alike). Long-term increases in synaptic AMPAR numbers, or in their conductances, make spines easier to activate, allowing EPSPs to build up voltage at faster rates and to higher magnitudes (see discussion on LTP mechanisms in the next section), which helps bAPs and dSpikes to better bring excitation to the soma, ultimately driving AP generation. However, overall changes in spine PSD AMPAR contents say very little about the true coding events happening in dendritic trees. And as both excitatory and inhibitory neurons are controlled by AMPAR-related excitation, not knowing in which cell type's spines the changes occurred makes it impossible to correctly understand their functional consequences. As we have explained earlier in this section, e.g. AMPARs containing only the GluA1 subunit are quite faster responders than GluA2-containing receptors, with a conductance difference of ~30 pS. The general idea that upon memory formation GluA1 is inserted into PSDs and later substituted by GluA2 during maintenance (Hong et al., 2013) might not work for all neurons: research has clearly shown that inhibitory interneurons do not, in general, have AMPARs with GluA2 subunits — which means they do not acquire GluA2 at *any* point: memory formation, maintenance, or expression. There seems to be no general biochemical panorama accounting for all neurons: which animal, which local region in the brain, which cell type, dendritic subunit, and particular microcircuitry, and which receptor composition, all contribute differences to the marvelous diversity nature has provided animals to respond to, and navigate through, their surroundings.

We studied the expression of the two most common subunits of AMPARs (GluA1 and GluA2) at BL neurons following recall 24 h after AFC, putting into context what the individual differences found in our rat population might computationally mean for their behaviors (G. Li et al., 2009), especially for fear generalization. By 2025, the precise distribution of AMPARs, or any of its subunits, on any one of the rat amygdala neuron types is still scarcely related, experimentally, to behavioral aspects of fear encoding. Thus, our finding that the higher GluA1 subunit expression occurs in the rat BL nucleus, the more the animal generalizes a cued-fear association (Moraes & Hardt, 2023) is of some importance, albeit

recognizably incomplete, and will have to wait for further elucidation of AMPAR precise dendritic distribution in this important brain area.

3.4 Synapses and plasticity

Where I explain some terms and knowledge of neuronal plasticity, with emphasis on long-term potentiation (which involves AMPAR function), and relate it to spine and dendritic phenomena.

Any changes in unmediated neuron-to-neuron communication are known as **homosynaptic plasticity**, and mostly affect *synaptic strength*, commonly measured as variations in EPSP amplitude. These changes may include *synaptic depression* (via presynaptic vesicle, or neurotransmitter depletion), *synaptic facilitation* (via residual calcium from previous stimulation, which increases the probability of vesicle release; e.g. experimentally induced by paired-pulse facilitation) (**Figure 3.4.1A**), and *post-tetanic potentiation* (PTP, via prolonged increases in presynaptic calcium availability) (**Figure 3.4.1B**), which range in duration between hundreds of milliseconds and several minutes (Johnston & Wu, 1994; Shepherd, 2004). Changes to synaptic strength that are not brought about by the neurons involved in a homosynaptic communication but by nearby neurons, are referred to as **heterosynaptic plasticity**. Examples of these include *presynaptic inhibition* and *presynaptic facilitation*, which mostly rely, respectively, on decreases and increases in the open probability of presynaptic calcium channels. Note that all these phenomena depend on the presynaptic vesicle release probability, regardless of the mechanisms influencing it (albeit mostly calcium-dependent), and probably happen at the same time among different synapses, perhaps to the same postsynaptic neuron. For example, moderately repeating pair-pulsing induces a higher steady-state amount of calcium at most of the involved presynaptic boutons, such that the arrival of more action potentials to any of them builds up from the already existent calcium concentration, which is elevated, thus *facilitating* the ensuing transmission. However, if the pair-pulses are too frequent, or too strong (such as in tetanic stimulation), or at least happen for a greater length of time than the presynaptic bouton can replenish synaptic vesicles with neurotransmitter, vesicle depletion occurs, and some synapses get *depressed* at the same time as others are still being facilitated or potentiated. This may happen at different rates among the several paired-pulse terminals, and so a balanced sum of these eventually results in small non-consequential current and voltage oscillations in the postsynaptic dendrites or may contribute to attenuate facilitation (**Figure 3.4.1B**). What all these processes have in common is that they are presynaptic, and, most importantly, result in short-lasting changes in EPSP amplitude (they are **short-term**), which

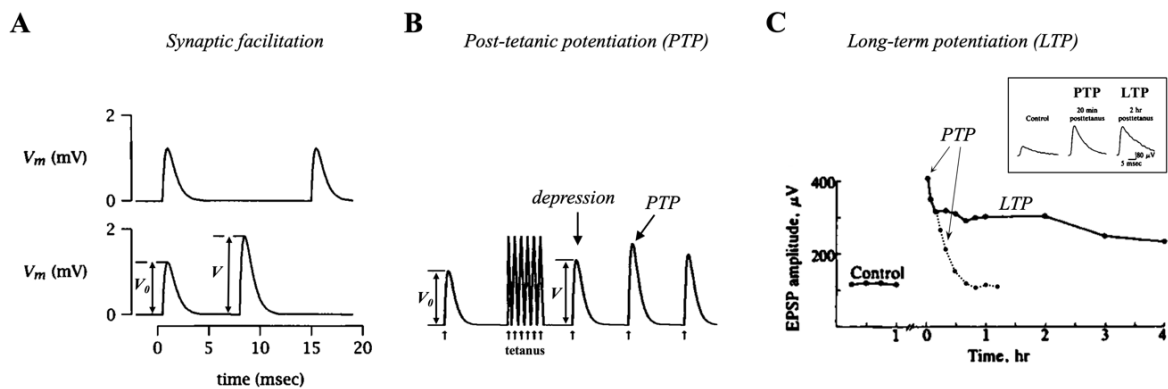


Figure 3.4.1. Some examples of functional short- and long-term plasticity. A and B show examples of short-term functional plasticity taken from recorded changes in somatic EPSPs. **(A)** The top membrane voltage (V_m) graph shows two closely spaced EPSPs (< 20 ms) still with the same amplitude (no apparent plasticity). The bottom plot shows the resultant increase in EPSP amplitude (before, V_0 , and after, V) after a stimulus is applied to an already *facilitated* neuron. **(B)** Following *tetanic potentiation* synaptic vesicles are mostly depleted, and the first test stimulus results in a somewhat meager increase in EPSP amplitude due to *depression*. However, calcium levels remain high in the presynaptic terminal, and a later test stimulus, once synaptic vesicles are refilled with neurotransmitter, reveals the functional PTP. **(C)** While the mechanisms behind facilitation, depression, and PTP are mostly presynaptic, postsynaptic influence allows for more durable changes in synaptic efficacy, of which an example is long-term potentiation (LTP). The inset shows three EPSP extracellular recordings: before stimulation, during PTP, and during LTP. The EPSP peak amplitude is plotted over time in the graph below, showing that for at least 4 hours the EPSP amplitude potentiation is kept high. Images adapted from Johnston & Wu (1994). A typical PTP long run trace in C was artificially added for demonstration.

either increases (homo- and heterosynaptic facilitation, and PTP) or decreases (depression, presynaptic inhibition) for some time. Naturally, the stimulus history of the synapses modulates these processes: if a synapse, for example, already departs from a state of increased release probability, further stimulation will depress it (as vesicles will be more readily depleted) rather than facilitate it. Also, until recently, these forms of plasticity have all been artificially induced, that is, they did not arise from naturally intrinsic biological (such as sensory) stimulation, but from experimental manipulation. Fortunately, there has been good indication that these short-term plasticity (STP) processes do indeed naturally occur, and recent data found that typical synaptic *efficacy* (EPSP amplitude change) varies close to 70% over time, and in many cases the fluctuations are well described by STP models, such as facilitation and depression (Ren et al., 2022). This is reassuring, given a moving life form needs constant change and updating of information from its surroundings.

It is tempting to envision a gradient of increasingly higher and persistent EPSP responses, as brains need longer-term retention, and starting with presynaptic-dependent mechanisms. But can these short-term EPSP amplitude enhancements be further extended in time? In the 1960s researchers found that PTP could also be “prolonged” (Lomo, 1968),

and later termed the phenomenon long-term potentiation (LTP, **Figure 3.4.1C**) (Bliss & Lomo, 1973), which seemed to greatly depend on stimulus intensity and frequency. These processes reflect changes to the EPSP shape, the EPSP itself being the sum of all the local backstage cellular and biochemical influences on the excitability of the neuron's membranes (or of many neurons' membranes, when extracellular field EPSPs are considered). LTP phenomena, however, don't seem to have a single dedicated mechanism, with some authors pointing to the existence of many LTP types (Malenka & Bear, 2004) (arguably including presynaptic forms (Enoki et al., 2009; Weichard et al., 2023)). LTP may indeed be regarded as a *continuation* of PTP, had the stimulus been given just a little bit stronger, or for a slightly longer period, engaging the proper postsynaptic mechanisms. Following a careful examination of LTP production in many important limbic forebrain structures (including the amygdala), Racine et al. found that the time course of LTP could be fitted by the sum of two reciprocal exponential curves with time constants of about 1.5 h (termed LTP1) and 5 days (termed LTP2), respectively (Racine et al., 1983). About two decades later, mounting evidence on the postsynaptic spatial role of calcium allowed a molecular explanation of Racine's LTP stage division: (a) **LTP1** requires post-translational modification of main synaptic proteins (including AMPA receptors, which are required for LTP establishment (Andrásfalvy et al., 2003; Y. Hayashi et al., 2000)) and activation of ryanodine receptors (RyRs; RyR3 is mostly expressed in spines, while RyR2 is expressed in the soma and neurites (Vega-Vásquez et al., 2022)), selectively elevating calcium availability to synapses; (b) **LTP2** requires activation of inositol-1,4,5-trisphosphate (IP₃) receptors (IP₃Rs) for further calcium release from intracellular stores (endoplasmic reticulum), specifically in dendrites (but IP₃Rs are also present in the soma), is protein synthesis-dependent, but does not rely on *de novo* transcription. Both LTP1 and LTP2 require NMDAR activation (as a calcium source, e.g., for RyR/IP₃R activation/inhibition (Hidalgo & Paula-Lima, 2024; Woll & Van Petegem, 2022)), and, given calcium itself activates RyRs and IP₃Rs, the whole process is known as *calcium-induced calcium release* (CICR)²³. Finally, a third, longer-lasting form of LTP was found, which came to be called (c) **LTP3**, selectively depending on L-type voltage-gated calcium channels (L-VGCCs), generating even more calcium influx, only partially requiring NMDAR activation, and requiring gene transcription (Raymond & Redman, 2002, 2006). Interestingly, this dependence on L-VGCCs was recently found to gate the mode of recycling endosome (RE) fusion with the plasma membrane (priming) in both spines and dendritic

²³ Just as a side note, keep in mind that although the expression of these two receptors is complicated by the several isoforms available and the differential expression patterns in different cell types (hippocampus vs cerebellum, for example), RyRs and IP₃Rs are mostly segregated into different subcellular locations, the most striking being dendritic spines and shafts, respectively (Sharp et al., 1993).

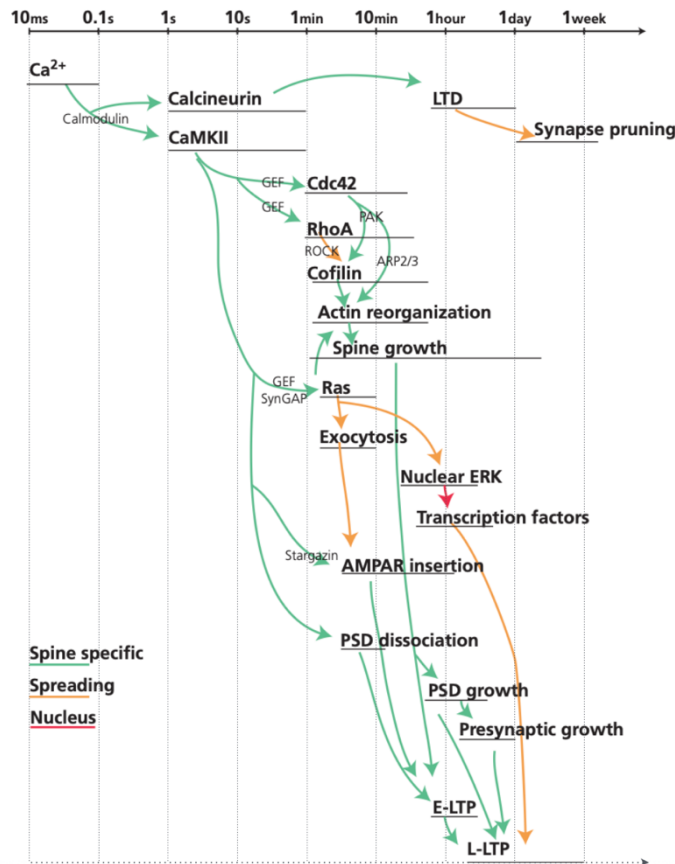


Figure 3.4.2. Temporal organization of biochemical processes conducting to LTP. From the moment an LTP-inducing stimulus is given to a dendritic spine, several biochemical cascades are sequentially engaged, including calcium elevation (10-100 ms), its integration by CaMKII and Calcineurin (1-60 s), AMPAR trafficking (1-10 min) and actin cytoskeletal rearrangements induced by small GTPases (1-30 min), plus presynaptic and PSD reorganization (minutes to hours), subserving the expression of Racine's LTP1 and LTP2, or early-LTP (E-LTP). Late-LTP (L-LTP) then corresponds to spine pruning and the transition between LTP2 and LTP3 (see main text). Image adapted from Stuart et al. (2016).

shafts, requiring NMDAR-dependent calcium to initiate RE fusion, whereas L-VGCCs regulate if primed REs collapse into the membrane or not; this is important because one of the membrane cargo proteins in REs are AMPARs (Hiester et al., 2017).

We have thus seen that a great deal of the *excitability* of neurons is supported by several biochemical and cellular processes (**Figure 3.4.2**). Most of these take place in spines along dendritic tree membranes, which should not come as a surprise given they represent almost 95% of the neuron's surface area (Rall, 1977). Hence, active dendritic conductances and backpropagation of action potentials (bAPs), including the generation of dendritic spikes, are essential to determine what happens to increased excitability of neuronal dendrites following the induction of LTP-like

phenomena (Frick et al., 2004). Classically used since the 19th century, and later applied to some extent to neuronal axons (Hodgkin & Huxley, 1952), *cable theories* have been used to interpret neuron electrotonic properties, including those introduced with compartmental models in the late 1950s and 1960s (Rall, 1977). Albeit outside the scope of this thesis, they have been used for decades as excellent models for neurons²⁴. Following many years of

²⁴ Some of these models can become rather complicated to solve analytically, and so the advent of computers has helped solve the ensuing differential equations numerically: computational neuroscience has since given us much insight into the integrative coding processes of neurons.

an axon-centric view, Rall was able to demonstrate that the most significant signal integration processes in neurons occur in dendritic trees, rather than in the soma. As most neurons in the CNS possess dendrites, this realization fundamentally changed our view of neural encoding. As we have seen, it would later be found that AMPARs mostly reside in dendritic

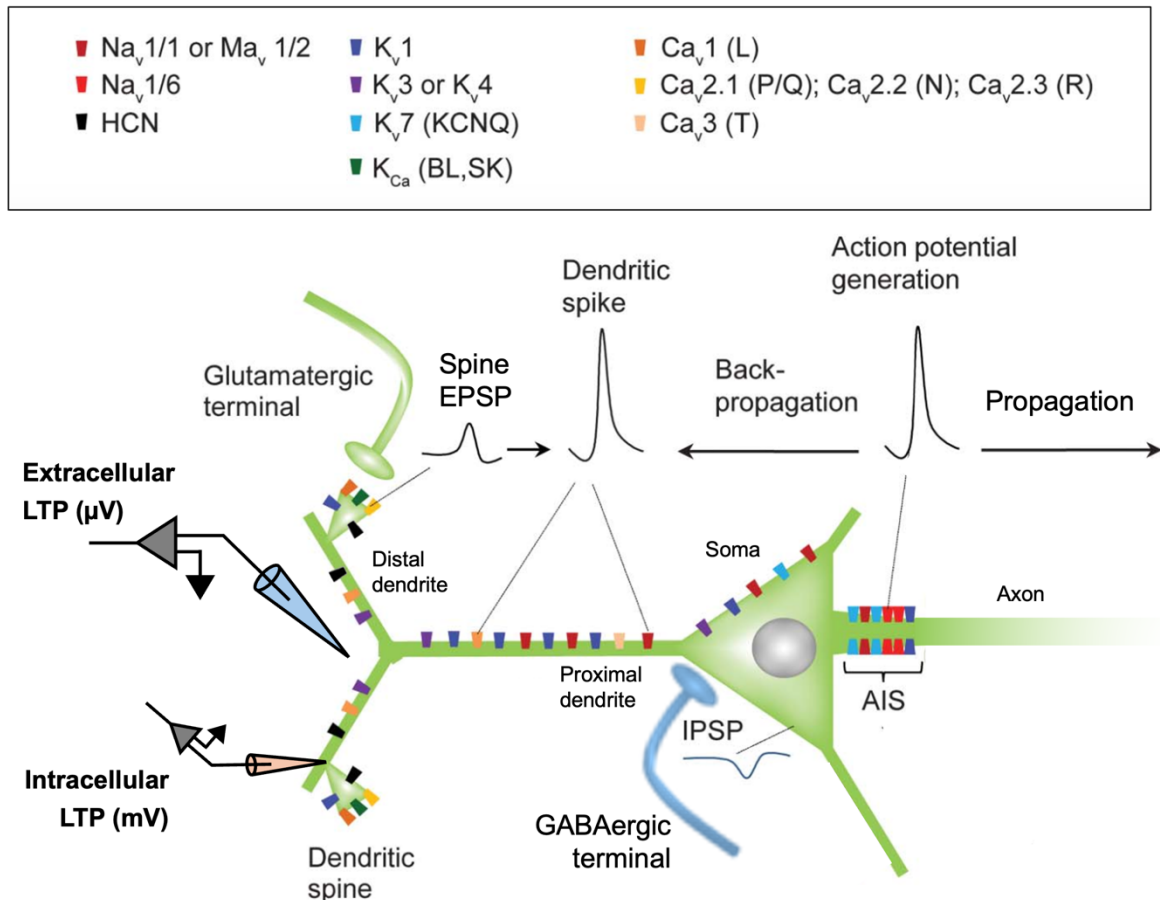


Figure 3.4.3. Overview of dendritic channel distribution within the functional context of EPSP generation. The scheme shows a pyramidal neuron truncated just after the axon initial segment (AIS), with its soma connected to a proximal dendrite bifurcating into two distal dendrites, each depicting a dendritic spine. The top left spine makes a synaptic contact with a presynaptic terminal from an excitatory (glutamatergic) neuron, generating a spine EPSP. At the lower right, an inhibitory (GABAergic) neuron makes a presynaptic contact with the postsynaptic soma, generating an IPSP. Summation at the AIS produces sufficient depolarization for the generation of an action potential, which also backpropagates (bAP) to the dendritic tree. Eventually, both the bAP, other incoming spine EPSPs, and active conductances along the dendrite help generate a dendritic spike, which here represents either a sodium- or calcium-spike (but may also be an NMDAR-spike). Also shown are two electrodes, displaying both extracellular and intracellular recordings of long-term increases in EPSP amplitude (LTP), along with their different magnitude scales. The inset on top summarizes in color-code some of the known ion channels expressed in the indicated neuron areas: Na_v , voltage-dependent sodium channel; K_v , voltage-dependent potassium channel; K_{Ca} , calcium-dependent potassium channel; Ca_v , voltage-dependent calcium channel; HCN, hyperpolarization-activated cyclic nucleotide-gated channel. Image adapted from Child & Benarroch (2014).

spines and directly contribute to the generation of synaptic signals. Rall also provided theoretical insight for how signals propagate along dendrites: since the *passive propagation* of electrical potentials along dendrites obeys a reciprocal exponential law over distance, the EPSPs arriving at the soma will not be of the same amplitude as those elicited at the synapse (Acker et al., 2016; Jayant et al., 2017): rather, they will be much, much smaller due to dendritic filtering. Inclusion of active conductances along dendrites further alters the magnitude distribution of dendritic EPSPs, which now largely depend on bAPs and dendritic spike generation, including the way they travel, and ultimately integrate, to influence the potential buildup at the axon initial segment (the site of AP generation) (Golding & Spruston, 1998). Now, as much as LTP is an important phenomenon, it is functionally observed in and structurally correlated to spiny synapses, not the soma (Harvey & Svoboda, 2007; Lang et al., 2004; Matsuzaki et al., 2004). And, again, between synapses and the soma there are the *dendrites*: plus, along the dendrites there are many, many different channels (Child & Benarroch, 2014) (a summary may be appreciated in **Figure 3.4.3**) with a plethora of distribution patterns (Larkum et al., 2022). I will not explore the resulting complexities of these added conductances, also because most of their functional expression and computational implications are still being worked out, but I wish to recall the two functionally different aspects of dendritic EPSPs I mentioned earlier: **dendritic spikes** (dSpikes) and **backpropagating action potentials** (bAPs) (see **Figure 3.4.4**). When an action potential is generated in the neuron AIS, it propagates bidirectionally: it follows its natural trajectory towards the axon terminal, *and* it travels back to the soma. From the soma, it spreads into the neuron's several dendrites, generating bAPs there, but for a short range: in the somato-dendritic axis there is a considerable influence of geometry (heavy dendritic branching), which passively attenuates bAPs (dendritic filtering; see **Figure 3.4.4**, right panel, **A** and **B**); furthermore, many neurons have somato-dendritic decaying distributions of Na_v channels (but not K_v channels), which also makes it difficult for bAPs to keep up (e.g. thalamocortical neurons of the dorsal thalamus (Williams & Stuart, 2000)). On the other hand, dendritic stimulation, especially at distal sites, spreads EPSPs locally, which quickly attenuate (see **Figure 3.4.4**, right panel, **C** and **D**), and if any potentials arrive at the soma, they are very small. Active conductances spread along the dendritic shafts thus help the meeting of bAPs and local EPSPs (even enhanced by LTP) by generating several types of dendritic spikes (G. J. Stuart & Spruston, 2015) (see **Figure 3.4.4**, left panel). The best studied so far are sodium spikes (generated by Na_v activity), calcium spikes (Ca_v activity) and NMDA spikes. Part of this spiking activity is computationally engaging and may indeed, some suspect, be the actual source of our neural codes (Poirazi & Papoutsis, 2020). Although the above discussion, for the sake of simplicity, considers the fate of isolate bAP or dSpike events, many excitable puncta might be active at the same time within a dendritic tree. Thus, the patterning of

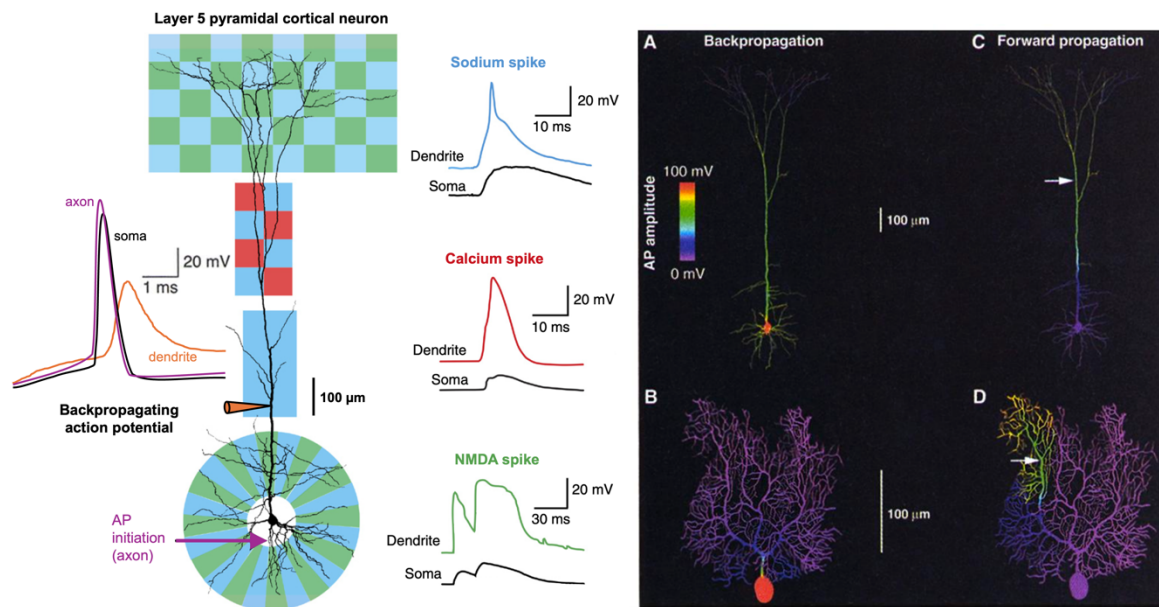


Figure 3.4.4. Sample recordings and simulations of dSpikes and bAPs. (Left) A neocortical layer 5 pyramidal neuron (central image) was stimulated at distal synapses to evoke an action potential (AP) at the AIS (purple trace), which backpropagated to the soma (black trace) and recorded as a bAP on a dendrite ~300 μm from the soma (orange trace). A weaker distal synaptic stimulation evokes three types of dSpikes: sodium spikes (blue trace and neuron areas), calcium spikes (red trace and neuron areas), and NMDA spikes (green trace and neuron areas). Note the different temporal scales. (Right) Model simulation of both backpropagation (A,B; soma stimulation) and dendritic spike forward propagation (C,D; location of synaptic stimulation indicated by white arrow) on a neocortical layer 5 pyramidal cell (A,C) and a cerebellar Purkinje cell (B,D). Notice the different patterns of voltage spreading. The image is an adapted composite from Häusser et al. (2000) (right) and Stuart & Spruston (2015) (left).

synaptic inputs gives rise to different integration strategies: some have been shown to be local (clustered), on the same dendrite, while others have been shown to be global (scattered), engaging different dendrites in the tree. The consequences of having one or the other integration mode are quite different: activation of clustered synapses most frequently gives rise to supralinear integration²⁵, while activation of scattered synapses often accounts for linear integration. The biophysical mechanisms (dendrite diameter, branching degree, input location, and active or passive properties) behind sublinear integration²⁶ are still not clear at this point, as more research is still needed (Tran-Van-Minh et al., 2015), but this strategy seems to also involve clustered synaptic inputs.

Locally, dendritic active conductances discussed above have a direct relationship with LTP phenomena, as they provide biochemical support for the latter. For example, at *distal* synapses from hippocampal pyramidal neurons, dendritic sodium spikes generated by Na_v

²⁵ That is, the dendritic *measured* EPSP is larger than the sum of the individual input-generated EPSPs.

²⁶ That is, the dendritic *measured* EPSP is lower in magnitude than the sum of the individual EPSPs.

channels are found to be needed for LTP (Y. Kim et al., 2015), as their inhibition blocks LTP. Basically, Na_v channel spikes induce an increase in intracellular calcium close to NMDARs and L-VGCCs, which as we have seen are essential for LTP induction and establishment. This finding articulates well with the LTP2 and LTP3 phases described above, given it facilitates RE fusion with postsynaptic membranes (Hiester et al., 2017), among other calcium-dependent effects. As a second example, another study found that concurrently to an LTP-induced increase in local dendritic excitability, there seems to be a shift of the inactivation probability of the channels eliciting A-type K^+ currents (which normally hyperpolarize this dendritic region) to higher values (they get inactivated faster), thus enhancing the amplitude of APs as they back-propagate (Frick et al., 2004). Accordingly, Kv4.2-KO mice have reduced dendritic CA1 hippocampal A-type K^+ currents and display enhanced induction of LTP via increased bAP amplitude and concurrent calcium influx (Chen et al., 2006), making yet for another example of the importance of ion channels in dendrites. Interestingly, Kv4.2 channels bind to PSD95 (Wong et al., 2002), as do many other synaptic plasticity-related PDZ motif-containing proteins, making them an important player in EPSP development. Many other examples abound, of course, accumulated along the last two to three decades.

LTP1 and LTP2 have put clocks on memories (or at least some of their subservient biochemical scaffolds), extending them between 1.5 hours and 5 days. But what is the extent of LTP3, or any “late-phase” of it? The problem with LTP is that it is a local experimental phenomenon, it is observed on, or close to single synapses, or a few close by synapses which have their excitabilities increased. But by its nature it does not seem to be a network phenomenon, as may be the case of dSpikes and bAPs, since LTP by itself is not an information entity but a process: a hard-rewiring, a structural and/or functional preparedness for communication, enabling routes to make it easier to flow — but it is not the communication itself. As memories are reconstructive (Easton et al., 2024; Spens & Burgess, 2024), their coding is of course dependent on the connection landscape and its wiring weights: LTP helps provide this landscape by biochemically shaping synaptic contacts, but it does not dictate the rules of signal integration we described above. An example is provided by the work of Nabavi et al., which have paired foot shocks with optogenetic stimulation of auditory inputs to the lateral nucleus of the amygdala, during a fear conditioning task²⁷: the “optical LTP” protocol mimicked an auditory stimulus and was shown, if delivered alone, to elicit the

²⁷ Fear conditioning is an experimental protocol whereby a subject animal with a patterned behavioral response (such as motor freezing) to a harmful, **unconditioned stimulus** (US, such as a foot shock), is repeatedly exposed to a neutral stimulus (such as a box, sound, or light) in conjunction with the harmful stimulus, so that the neutral stimulus becomes **conditioned** (CS) to the US, and the animal reacts to presentation of the CS as if it was the US.

fear response obtained after pairing with the foot shocks (Nabavi et al., 2014). To achieve this, the authors artificially expressed a light-activated ion channel (ChR2) in the medial geniculate nucleus and auditory cortex: these, making turns with endogenous AMPARs, provided the excitability landscape we discussed before, making it possible for more efficient communication along the routes involved in expressing the fear memory. Additionally, heterosynaptic phenomena, the influence of nearby glial cells, namely astrocytes, and the extracellular matrix, are but a few other influencers of whether a particular signal *goes* or *does not go*, which may ultimately decide if an animal experiences a particular memory or not. A pure LTP3 extension magnitude would mean that *that synapse* or a small *set of synapses* keep strengthened without any other influences for a long, long time. But who is to say (or experimentally check) that, inside its home cage, whatever the rat is doing over a year, waiting for the experimenter to re-stimulate its synapses and see if the EPSP is still augmented or not, those synapses have not been visited once in a while by a dSpike or a bAP, or that some change in the dendritic content of some nearby voltage-gated sodium channel has not occurred? Could we still say that *that LTP* we saw during the first hours or days is the same we have induced, and the one that is still showing up a year later? There are indeed some reports of very long-lasting LTP observations, up to one year in one animal using a high frequency stimulation (HFS) protocol in the hippocampal dentate gyrus, with four sets of five trains of 100 Hz, 1 s inter-train intervals. Still, it decays (albeit very slowly) and it seems simple exposure to an enriched environment even 14 days after induction may indeed reverse it (Abraham et al., 2002), meaning that re-routing of synapses or synaptic resources to other memory patterns, just by letting an animal experiment with its surroundings, interferes with the original landscape — and this is certainly the natural situation for most animals. It would be biologically forbidding to keep every neuron with input information from the environment forever heightened. The same author ran a literature survey in order to estimate the average duration of LTP3 and found that, at least in the dentate gyrus, the average decay time was ~20.3 days, which is a little less than 1 month (Abraham, 2003). I very much doubt that any pure form of LTP can last for a lifetime by itself, as it is commonly alluded. Humans are biased in many respects: cognitively, we have evolved a “brain network” called society. This network allows repetitive recollection of events and experiences at a level no other animal has — by continuous and controlled re-exposure through storytelling, books, many forms of gathering, and a plethora of art forms. From an early age, we collectively retell extraordinary events of ours, and past lives, be it in the form of historical events or simple anecdotes. No other animal does that. This, of course, may create the *illusion* of very long-term memories: over many years, the story of some event is retold many times, meaning the brain areas, the local dendritic subunits and microcircuits involved in the experience of that event’s settings have been reactivated (and possibly updated) over

and over. This is not a surprise, given a slowly decaying LTP trace can be re-potentiated, which seems to have something to do with keeping AMPA receptors in synapses (Z. Dong et al., 2015). Also, within our kingdom, we are not only, as Primates, at the tip of the maximum lifespan (if we exclude cetaceans; **Figure 3.4.5**), but within our own order we, at 122.5 years, are quite far from the second place, occupied by the chimpanzee (*Pan troglodytes*), at 68 years, and certainly farther from the average for Primates, at ~30 years (de Magalhães et al., 2023). Without our cognitive abilities, longevity and socially driven repetition of life events, it is difficult to imagine why would nature provide animals with a mechanism to indefinitely over-excite any organized representation of stimuli. Also, generation of very long LTP3 such as the one in the Abraham et al. (2002) study requires very strong induction protocols, such as HFS, which do not quite resemble the physiological scene (Raymond,

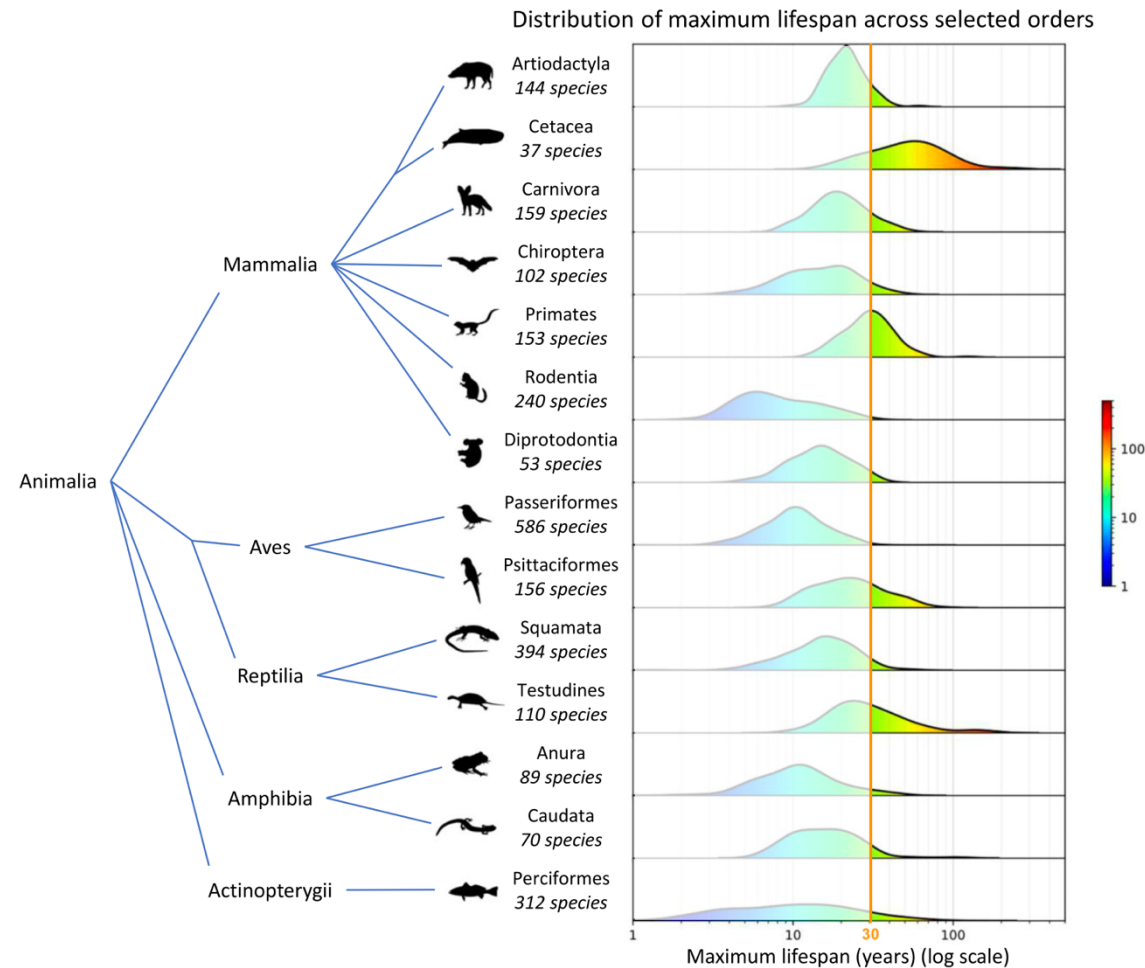


Figure 3.4.5. Distribution of maximum lifespans for several orders within the *Animalia* kingdom. The number of species for each order is indicated below the order name. As an example, among the 153 species of Primates considered, some have reached a maximum of 13 years (*Arctocebus calabarensis*), while others have reached up to 122.5 years (*Homo sapiens*). The *Cetacea* order is the only animal group having an average maximum lifespan of over 30 years, also having the oldest record for an animal, at 211 years (the bowhead whale, *Balaena mysticetus*). Adapted from Magalhães et al., 2023.

2007). An organism's "hardware" may provide the means for an extreme goal (such as a lifetime "pure" LTP phenomenon), but that does not mean that this goal serves with any relevance that organism's nature, wellbeing, or survival needs.

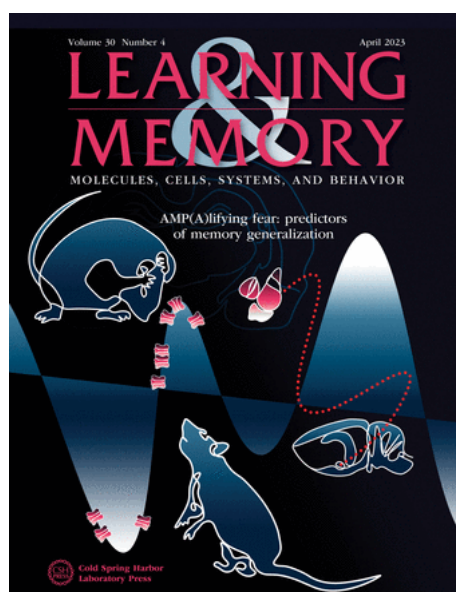
I truly believe that part of the future of neuroscience research — in this case, already part of the present — lays in a serious combination of cellular and molecular biology with mathematics and computational modelling, which is already at play. We may have a great deal of the knowledge we need spread among millions of research papers, but the dream of a coherent picture, a *reproducible* picture of individual organism's behaviors, individual brain's local regions, individual neurons within those regions, and even individual dendritic subunits therein, is certainly not one that, quite paradoxically, our self-studying brains are able to grasp without external aid. Modelling of dynamical systems by way of differential equations, which produce not one answer, not one single behavioral or molecular path, but a landscape of them, may quite well be how our scientist descendants will ultimately tackle their inner self-computational diversity.

4 Research data

4.1 Foreword

The following data was submitted to *Learning & Memory* (and published under doi: 10.1101/lm.053612.122) with the title “*Specific behaviors during auditory fear conditioning and post-synaptic expression of AMPA receptors in the basolateral amygdala predict inter-individual differences in fear generalization in male rats*”. The authors, PhD candidate’s advisor Dr. Oliver Hardt, and the candidate Bruno José Moraes, were kindly invited by Associate Editor Susan Cushman to have their work featured in the cover of the journal’s April 2023 issue. We are deeply thankful to the workart of the illustration designed by MD and artist Sofia Vilar.

Below we reproduce both the cover and the accompanying text.



During auditory fear conditioning, rodents, such as rats, are trained to fear a tone by pairing it with an electric footshock in a conditioning chamber. Although rats readily learn this association, they often also become fearful of environments that resemble the conditioning chamber. Moraes and Hardt (*LearnMem* 74–84) explore behavioral and biochemical parameters that might predict whether rats will develop this type of fear generalization. They found that certain behaviors during training, such as freezing and rearing, as well as the expression of AMPA receptors in the amygdala, can account for the extent of fear generalization. The cover art shows a sound wave, referring to the tone used in auditory fear conditioning, with crests and troughs reminiscent of amygdala synapses and their embedded AMPA receptors. As the rats on the cover illustrate, the larger number of AMPA receptors in crests is associated with freezing during training (which leads to stronger generalization of fear), while the lower number of AMPA receptors in troughs refers to rearing and exploring the environment (leading to less fear generalization). Finally, the coronal cut shows the location of the amygdala in a rat brain.

4.2 Publication introduction

Fear expression of rats in auditory fear conditioning (AFC) usually varies across an experimental cohort although all individuals have been exposed to the same stimulus type, display little intraspecific genetic variation, and have experienced similar controlled, uniform living conditions. Fear is usually assessed by measuring freezing, the canonical behavioral response in these tasks. While rats express freezing in a variety of natural situations and often as just one response among others to threat, in experimental procedures in laboratory settings it is the typical behavioral response when flight or attack do not effectively diffuse the perceived threat, as is the case in most auditory fear conditioning protocols where foot shock cannot be escaped. A common observation in these laboratory tasks is that the same fear conditioning training protocol gives rise to broad variance of fear expression during subsequent retrieval or extinction. For example, rats may present anywhere between fast and slow reduction of freezing, and some rats may even show no extinction at all after twenty extinction sessions (Galatzer-Levy et al., 2013).

Such variations in the freezing response may not be entirely random and could reflect to some extent intervening organismic variables. For example, different mRNA profiles of principal neurons in the rat nucleus accumbens correlate with individual differences in addiction-like behavior (Imperio et al., 2016). Similarly, inter-individual variations in stress-related responses have been linked to individual differences in the expression of fear and anxiety (Kazlauckas et al., 2005; Bush et al., 2007; Shumake et al., 2014; Walker et al., 2008). Analogous parameters also influence conditioned fear memory, leading, for example, to differences in overall levels of freezing or in the extent of generalized fear (Rudy & Pugh, 1996; Baldi et al., 2004), which the intensity of the CS and the number of conditioning trials can moderate (Desiderato, 1964).

Another source accounting for variance in this fear response may arise from behaviors other than freezing that animals express in these tasks. For example, placing a novel object into a previously explored open field can result in both low and high exploratory activity in mice, which can predict their level of anxiety expressed in an elevated plus maze (Kazlauckas et al., 2005). In AFC, candidate behaviors that might help explain variance in freezing are grooming and rearing. While grooming has generated some contradictory results in predicting fear expression (e.g., Reimer et al., 2015), it has been shown that rodents express rearing when their fear is low (Gray & McNaughton, 2000). Rearing is part of the exploratory behavior rodents exhibit when acquiring spatial-contextual memories. Low rearing in stressful situations therefore could indicate that animals sample the environment less, resulting in spatial-contextual knowledge insufficient for accurate context discrimination. This, in turn, could lead, for example, to higher generalization of contextual fear.

While some degree of fear generalization is adaptive, alerting animals to threats resembling previously encountered ones, excessive generalization can promote anxiety in otherwise safe environments (Xu & Sudhof, 2013). Cued fear memory is known to generalize over time and, in AFC, this process involves the interplay between the medial geniculate nucleus (MgN) of the thalamus, and the amygdala (Ferrara et al., 2017). The amygdala is critically implicated in fear memory and expression, and its basolateral (BL) nucleus contributes to the ability to discriminate between aversive and non-aversive stimuli (Tovote et al., 2015). Different populations of neurons within the BL nucleus encode generalized and cue-specific associations (Ghosh & Chattarji, 2015; Grosso et al., 2018). Interestingly, increased neuronal activity in the BL nucleus has been shown to promote fear generalization (Rajbhandari et al., 2016; Ferrara et al., 2017). Most excitatory transmission in the mammalian brain is mediated by AMPA receptors (AMPA), ionotropic channels that are differentially composed of four subunits (GluA1-4). The expression of the GluA1 subunit, in particular, increases in the rat amygdala during fear memory consolidation (Hong et al., 2013). Thus, inter-individual differences in GluA1 expression in the BL nucleus might account for differences in fear generalization.

Here, we used a standard AFC protocol to seek out variables that may explain differences in fear responding. We trained male rats with two tone-shock (conditioned stimulus, CS and unconditioned stimulus, US) pairings. When testing the response to the CS in a familiar testing context (Context A) different from the training context (Context B), we observed a wide range of freezing to the known testing context alone (assessed as Pre-CS freezing), which is a common outcome of this standardized procedure. The rats, however, had not expressed generalized fear during initial exposure to the training context, before they had experienced the first tone-shock pairing, therefore making the generalization during test an outcome of fear conditioning. To identify possible behavioral and biochemical predictors of this generalization, we used correlational and cluster analysis on several behavior parameters, as well as measurement of AMPAR expression at BL nucleus' synapses. We found that low rearing and high freezing during fear conditioning, as well as increased expression of GluA1-containing AMPARs in the BL nucleus, significantly predicted generalized fear to the familiar testing context.

4.3 Materials and methods

Subjects

We used 23 male Sprague-Dawley rats (Charles River, QC, Canada) weighing 250-300 g at the time of arrival. Two independent animal cohorts were used for two AFC learning

replicates (R1 and R2, see Results section). Original sample size was $n = 12$ for both replicates (total $n = 24$) but one animal from R2 was dismissed for the present study since it was an identified outlier (Grubbs test, $Z = 3.11$, $p < 0.01$; 47.1% freezing at acquisition timepoint TS1, please see below for details). Rats were housed in pairs inside $26.7 \times 48.3 \times 20.3$ cm Plexiglas cages (Ancare, Bellmore, NY, USA) with environmental enrichment provided by PVC hollow cylinders, 10 cm in diameter. Lights in the animal colony turned on at 7:00 AM and went off at 7:00 PM. Animals were tested during the lights-on phase. Food and water were provided *ad libitum*.

Behavioral Procedures

Handling and habituation. Upon arrival, we handled rats in the colony for 7 consecutive days. This was followed by 3 consecutive days of habituation to the testing box (Context A) in which we would later assess conditioned auditory fear (**Figure 4.4.1A**). Context A consisted of a $30 \times 25 \times 30$ cm Plexiglas box (Coulbourn Instruments, Whitehall, PA, USA) with a white plastic floor and arched white Plexiglas back walls, incandescent house light (5 lumens), and a distinct odor (30% peppermint solution). A camera mounted on top of the box recorded the animals. The testing box was housed inside a sound-attenuating chamber with a fan that provided ventilation. A set of four such chambers was placed inside a bright illuminated room. For each of the 3 days of habituation, we placed rats inside Context A for 5 min, after which we returned them to their colony.

Auditory fear conditioning. Rats were fear conditioned inside the training context (Context B), a $30 \times 25 \times 30$ cm Plexiglas box with a metal grid floor for shock delivery (Coulbourn Instruments, Whitehall, PA, USA), illuminated in red light (dark environment), and distinguishable further by an odor (30% vanilla solution). The box was housed in a sound-attenuating chamber, ventilated with a fan. A set of four such chambers was placed inside a dark testing room, attached to a dark antechamber. After transportation from the colony, we held animals in the antechamber for 5-10 min before the procedure started. Once rats were placed into a box, the first tone (4 kHz, 75 dB, 30 s) was presented after two minutes (Pre). It co-terminated with a foot shock (0.75 mA, 1 s; TS1). Ninety seconds later (ITI, intertrial interval), the second tone-shock pairing was administered (TS2). Sixty seconds later (Post), animals were removed from the box, returned to their home cages and back to their colony (see **Figure 4.4.1A** and **Figure 4.4.2A** below). A camera mounted on the front wall of the conditioning box recorded the sessions for later analysis.

Fear memory retrieval (test). We tested rats in Context A. After transportation from the colony, animals were immediately placed individually inside a testing box, where they remained for a total of 3.5 min. After 2 min in the testing box (Pre-CS), a 4 kHz (75 dB) tone was played for 30 s (CS). We removed the animals 60 s later.

Animal sacrifice

We sacrificed animals 5 min after the test session. We induced deep anesthesia by placing individual rats inside an isoflurane-gas filled desiccator for no more than 2 min. Then we quickly decapitated the rat. We collected brains and put them into small metal-mesh cups, which we immediately immersed into liquid nitrogen for ~20 s, and then wrapped the brains with aluminum foil. The frozen brains were then stored at -80°C until further processing.

Tissue collection

We collected amygdala tissue inside a cryostat-microtome (Microm Instrumentations), punching through coronal sections (**Figure 4.4.6** below) with a 1-mm Neuro Punch (Fine Science Tools) at the amygdala, next to the external capsule, located approximately from bregma -1.80 to -3.96 mm, interaural 7.20 to 5.04 mm (Paxinos & Watson, 2004). Cryo-sectioning and punching were performed at -20°C , with tissue collected into vials containing homogenization buffer (30 mM Tris-HCl (pH 7.5), 1 mM EGTA, 4 mM EDTA, 2 mini-tablets of EDTA-free Complete protease inhibitors, Roche) on ice and homogenized with a small hand homogenizer.

Differential centrifugation for post-synaptic density (PSD) isolation

All centrifugation steps were carried out at $+4^{\circ}\text{C}$. Briefly, samples were centrifuged at $500 \times g$ for 10 min to remove nuclei and cell debris, and the supernatant further centrifuged at $100\,000 \times g$ (Beckman Coulter) for 60 min to separate the cytosol (supernatant) from membranes and organelles (pellet). The pellet was re-suspended in Triton-X100 0.5%, incubated at room temperature for 20 min, applied over a sucrose cushion (1 M sucrose, 1 mM EGTA, 4 mM EDTA, pH 7.4) and further density-centrifuged at $100\,000 \times g$ for 60 min. Forty to 60 μL supernatant were stored at -20°C as a Triton-X100-soluble fraction (extra-synaptic fraction), with the remainder discarded and the pellet re-suspended in RIPA buffer and stored at -20°C as the Triton-X100-insoluble fraction containing the post-synaptic density (PSD) proteins (post-synaptic fraction).

Protein quantification

Total sample protein was determined using the bicinchoninic acid (BCA) assay (Pierce). In brief, 200 μL of working reagent were added to 2-8 μL of sample and incubated at $+37^{\circ}\text{C}$ for 30 min. Absorbance was read at 565 nm in a microplate reader and sample protein concentration estimated by interpolation from a bovine serum albumin (BSA) standard

curve. Laemmli sample buffer was added to all original samples, and these were stored at -20°C until later use.

SDS-PAGE and immunoblotting

For normalization purposes, we loaded the same amount (3 μg) of total protein from each sample into either 8% or 10% T polyacrylamide gels and ran them at constant voltage (60 V for ~ 40 min, plus 120 V for ~ 1 -2 h). We transferred proteins onto PVDF 0.45 μm membranes at constant current (300 mA) for 1 h and immunodetected them using specific primary antibodies against GluA1 (1:1000, Millipore) and GluA2 (1:1000, Millipore) subunits (~ 1 -2 h incubations), and an appropriate HRP-conjugated secondary antibody (1 h incubation). Signal was detected by chemiluminescence with an ECL kit (Pierce), and membranes scanned using a Storm Laser scanner (Molecular Dynamics).

Data analysis

We measured freezing levels using FreezeView software with an 0.25 bout and an average 3.5 signal threshold. We manually confirmed the accuracy of the automatic measurement. An experimenter blind to condition measured rearing and grooming levels manually. We defined rearing as animals rising above half video screen, either solely supported by their hind legs or having their paws against the glass walls. We defined grooming as animals showing any of the four syntactic phases (elliptical, small unilateral and large bilateral strokes, and body licking). For each behavior, we measured the number of bouts and their duration. Although most literature reports behavior duration as phase percentage, we also decided to analyze data without transformation to avoid scaling effects. Immunoblotting bands were analyzed and quantified using ImageJ software. For statistical analyses we used GraphPad Prism (D'Agostino & Pearson omnibus K^2 normality test, Kendall's coefficient of concordance test, Friedman's test with Dunn-Bonferroni multiple comparisons post-hoc tests, Wilcoxon matched-pairs signed rank test) and IBM® SPSS® statistics software (Student's t test, Mann-Whitney U test, Shapiro-Wilk normality test, Pearson and Spearman correlations, cluster analysis and *post-hoc* power analysis). Specifically for cluster analysis, we used squared Euclidean distance as distance measure between cases, along with the between-groups linkage clustering algorithm, also called average linkage method, which is the most stable to estimate cluster number and size (Yim & Ramdeen, 2015). Since we intended to probe the occurrence of 'behavioral' clusters, we used the same type of measure for all cases in the analysis: we chose duration in seconds because we did not include the grooming bout count in the study, thus precluding the use of grooming average bout duration and absolute bout frequency data (see **Figure 4.4.2A**). Unless otherwise stated,

we report mean $\pm$ 1 SEM. Except for Shapiro-Wilk normality tests, the null hypothesis was rejected whenever $p < 0.05$.

4.4 Results

Broad range of Pre-CS freezing responses during fear memory test

We conducted a standard auditory fear conditioning protocol, consisting of three consecutive days of habituation to the testing context (Context A), then, one day later, an auditory fear conditioning session (Context B), and finally on the next day a long-term retention test (Context A) (**Figure 4.4.1A**). During test, animals were allowed to explore the box for two minutes (Pre-CS phase) followed by presentation of a 4 kHz tone for 30 seconds (CS

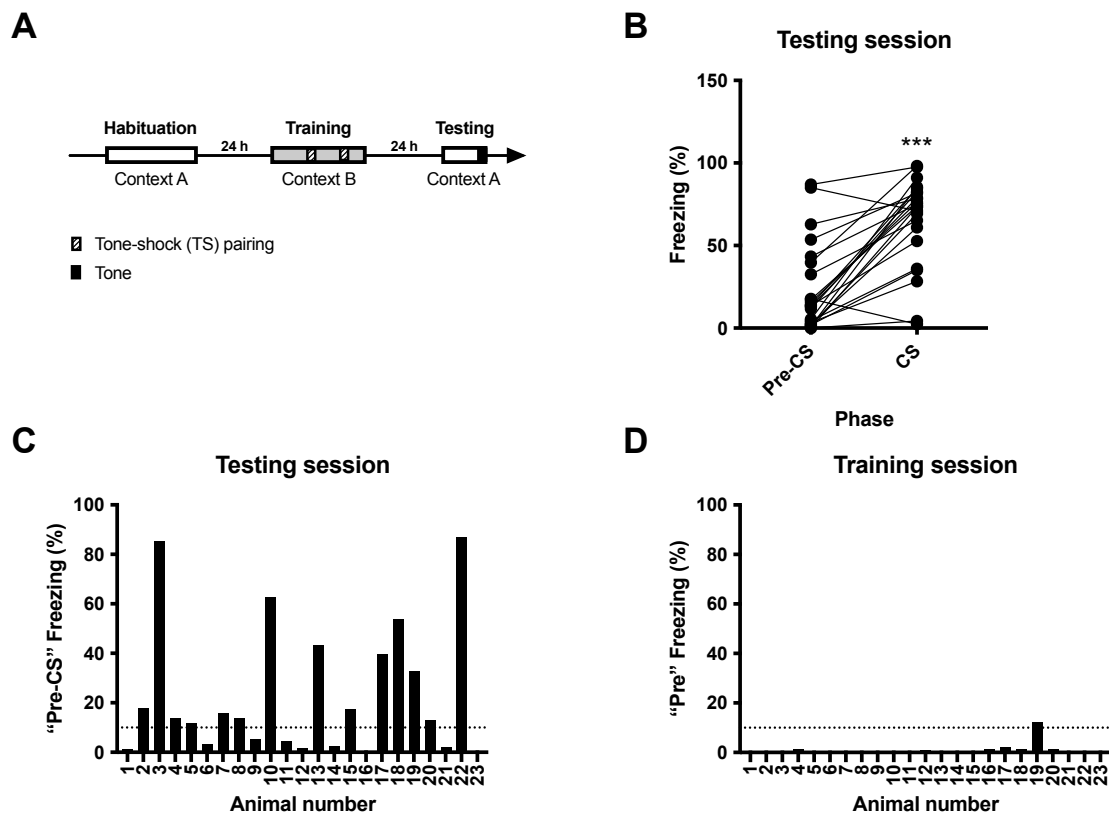
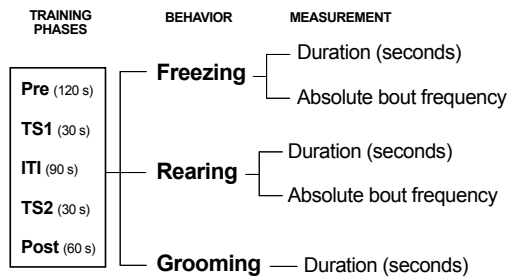


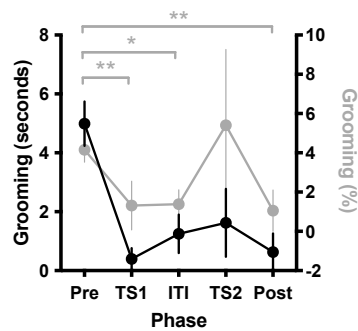
Figure 4.4.1. Wide range of fear responses to the testing context following fear conditioning. (A) Schematic representation of the experiment. Animals were habituated to context A (Context A) for 3 days (5 min/day), received two tone-shock pairings in context B (Context B) and were tested by presenting the tone, without shock, in Context A, 24 hours later. **(B)** Average individual freezing before (Pre-CS) and during tone presentation (CS) during the testing session; Wilcoxon matched-pairs signed rank test: Pre-CS vs CS, $W = 262$, $***p < 0.001$. **(C)** During the 2 minutes preceding presentation of the tone (Pre-CS phase) in Context A, there was an overall unexpected widening of fear responses to the context. Horizontal, dashed lines indicate cut-off at 10%. **(D)** Freezing (in %) to Context B before the first tone-shock pairing (Pre phase) at the training session was minimal. Same cutoff as in (C) is indicated.

phase). We measured freezing duration over both phases. Based on previous findings of others indicating an average baseline of freezing below 20% (see, e.g., Watson et al., 2016), as well as our current results showing that animals displayed on average 0.9% freezing during the 2-min “Pre” training phase, we set the baseline freezing threshold to 10% for our analyses. Because both Pre-CS and CS variables seem to be inconsistent with a Gaussian distribution (D’Agostino & Pearson omnibus K^2 test: Pre-CS, $K^2 = 8.19$, $p = 0.0166$; CS, $K^2 = 6.38$, $p = 0.0412$; see also **Table S2**), we analyzed behavior during test using a Wilcoxon matched-pairs signed rank test and detected a significant effect (Pre-CS vs CS, $W = 262$, $p < 0.001$; **Figure 4.4.1B**). We noticed, however, that the coefficient of variation was almost three times higher for Pre-CS (116.5%) when compared to CS phase (41.17%). Indeed, Pre-CS freezing responses varied considerably, with over 60% of the animals freezing above 10% of the 2 min Pre-CS phase time (**Figure 4.4.1C**). We did not find such variation in freezing to the context prior to the first shock for the training session (average Pre < 1%; **Figure 4.4.1D**; only 1 animal froze above 10% of the 2-minute Pre-CS phase, namely 12.3%). Thus, animals did not present with pre-existing fear to a novel context (Context B) and, following auditory fear conditioning, a considerable number of animals generalized their fear to a known context (Context A).

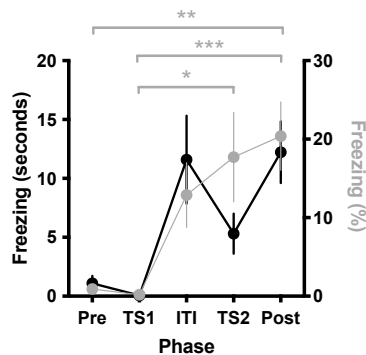
A



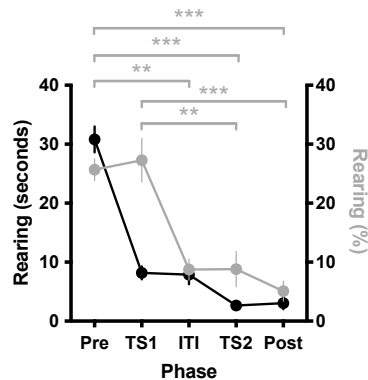
B



C



D



(See caption on next page.)

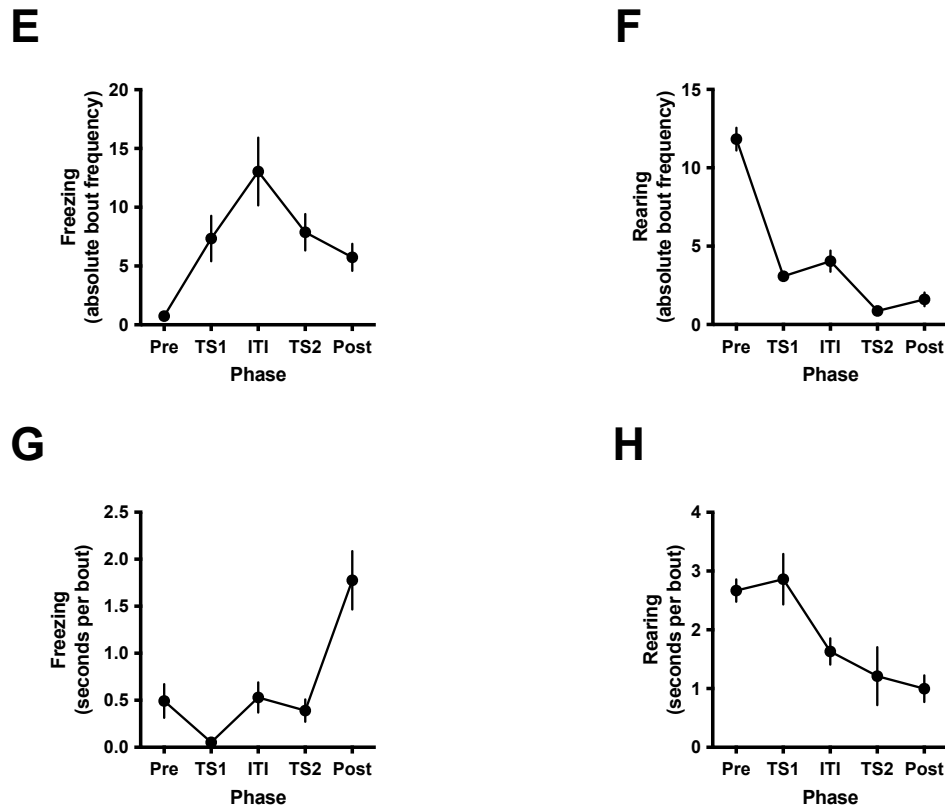


Figure 4.4.2. Behavioral profiles during fear memory conditioning. [Previous page:] (A) We split up the auditory fear conditioning session into five phases (“Pre”, before conditioning; “TS1”, first tone-shock presentation; “ITI”, intertrial interval; “TS2”, second tone-shock presentation; “Post”, after conditioning) with different durations (in seconds), and measured for each phase both absolute bout frequency and time spent freezing and rearing, as well as time spent grooming. (B–H) Behavior profiles included time spent grooming (B), freezing (C) and rearing (D), [This page:] absolute bout frequency of freezing (E) and rearing (F), as well as average bout duration of freezing (G) and rearing (H). Freezing, rearing and grooming were recalculated as phase percentage for phase comparison (B–D, gray traces). For the latter we ran a Friedman’s test with Dunn-Bonferroni multiple comparisons test as post-hoc analysis ($p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). All data points represent mean $\pm$ 1 SEM.

Canonical analysis suggests that characteristic training behaviors do not predict fear generalization

To identify behavioral characteristics observed in the training session that could predict the generalized fear to the test context (Context A), we considered three well-characterized and easily identifiable behaviors in the rat – freezing, rearing and grooming (Cain & LeDoux, 2007) – for which we measured duration in seconds and bout number, and calculated average bout duration in seconds per bout (**Figure 4.4.2A**). Expression of these behaviors during the training session varied across phase transitions (**Figure 4.4.2B–H**), including “Pre” (before conditioning), “TS1” (first tone-shock presentation), “ITI” (intertrial interval),

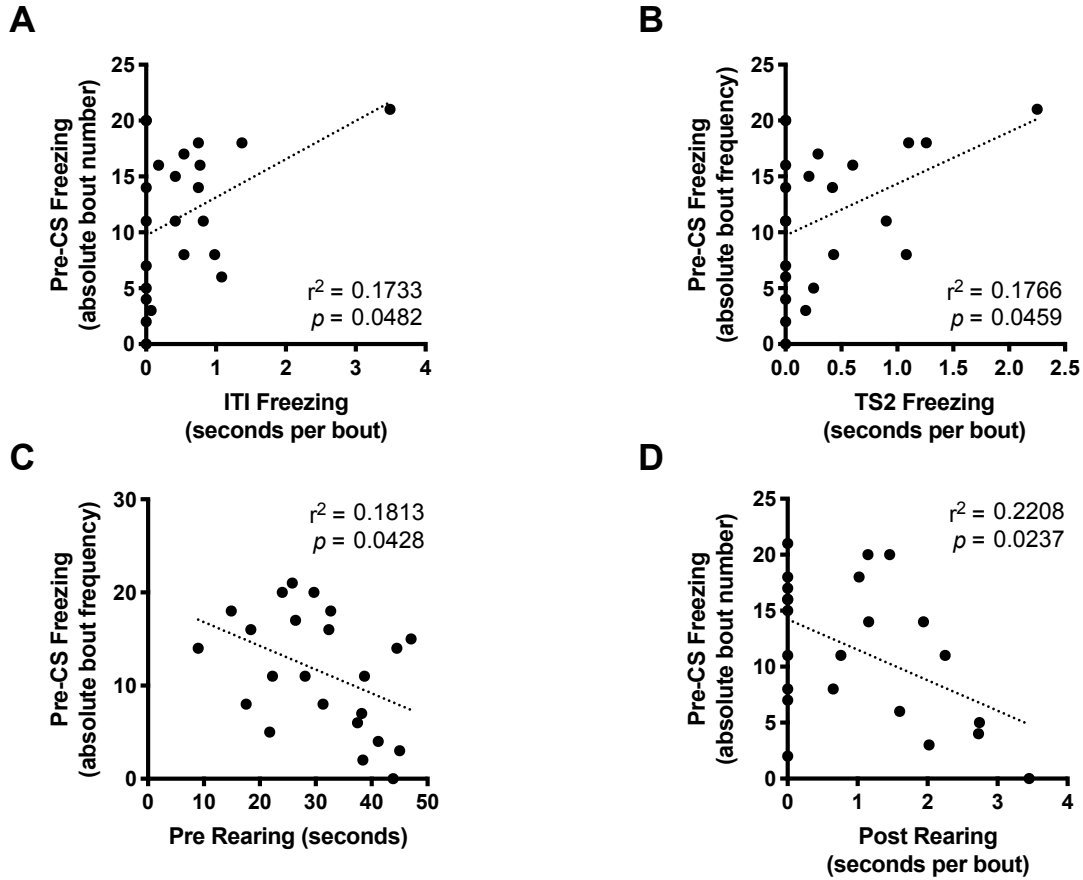


Figure 4.4.3. Training parameters poorly predict Pre-CS freezing during test. Only four out of 120 Pearson correlations were statistically significant. Training ITI Freezing (**A**), TS2 Freezing (**B**) and Post Rearing (**D**) average bout duration (in seconds per bout), and Pre Rearing duration (in seconds) (**C**) linearly correlated with testing absolute bout frequency of Freezing parameter during Pre-CS phase. Squared Pearson coefficient (r^2) and p -value indicate that only between 17-23% of the Pre-CS Freezing bouts during testing can be explained by any one of these four training parameters.

“TS2” (second tone-shock presentation) and “Post” (final phase following conditioning). Because each phase had a different duration (**Figure 4.4.2A**), direct comparison between phases was not possible without normalization, and this is only plausible for duration measurements, not number of bouts. We therefore transformed training parameter phase durations (in seconds) into phase percentages for grooming, freezing and rearing (**Figure 4.4.2B-D**, gray traces). Because only Freezing “Post” ($K^2 = 3.38$, $p = 0.184$), Rearing “Pre” ($K^2 = 1.25$, $p = 0.536$), “TS1” ($K^2 = 1.65$, $p = 0.438$) and “ITI” ($K^2 = 4.32$, $p = 0.115$), and Grooming “Pre” ($K^2 = 1.16$, $p = 0.560$) were normally distributed (D’Agostino & Pearson omnibus K^2 normality test), we performed a non-parametric Friedman’s test on each transformed parameter with Phase (Pre vs TS1 vs ITI vs TS2 vs Post) as repeated factor, as well as a related-samples Kendall’s coefficient of concordance test for effect sizes evaluation. The test detected a significant (Freezing, $\chi(4)^2 = 34.64$, $p < 0.001$; Rearing, $\chi(4)^2 = 39.85$, $p < 0.001$; Grooming, $\chi(4)^2 = 32.83$, $p < 0.001$) and moderate effect size (Freezing, $W =$

0.377; Rearing, $W = 0.433$; Grooming, $W = 0.357$) of Phase for each measure, whilst post hoc Dunn-Bonferroni analyses confirmed within-phase differences (**Figure 4.4.2B-D**, gray traces). We next explored whether any of the behavioral parameters or a combination of these could explain variability of Pre-CS freezing (overall duration in seconds, absolute bout frequency, and average bout duration in seconds per bout). Because only 15 of the 41 training parameters (~ 37%) were normally distributed (assessed via D'Agostino & Pearson omnibus K^2 normality test; see supplementary **Table S1** and **Table S2**), we used both parametric (Pearson) and non-parametric (Spearman) correlational analysis, along with graphical inspection of variable relationships. Within the 40 possible Pearson correlation tests between test session Pre-CS parameters and each of the other training and test behavioral data (supplementary **Tables S3-S5**), our analysis detected no linear relationships ($p > 0.05$) when comparing training session parameters against the total duration of Pre-CS freezing during test and the average duration of Pre-CS freezing bouts. We detected medium-sized

Table 4.4.1. Summary of significant ($p < 0.05$) correlations between the indicated training phase parameters and 'Pre-CS freezing absolute bout frequency' (see **Figure 4.4.3**). Pearson statistic (r^2) and p-value are shown. Parameter duration is in seconds; bout duration is in seconds per bout.

Acquisition Parameter	Phase	Pearson r^2	p-value
Freezing bout duration	ITI	0.173	0.048
	TS2	0.177	0.046
Rearing duration	Pre	0.181	0.043
Rearing bout duration	Post	0.221	0.024

linear correlations between Pre-CS freezing bout frequency during test and (a) the average ITI freezing bout duration, (b) the average TS2 freezing bout duration, (c) the total duration of rearing during Pre, and (d) the average Post rearing bout duration ($0.17 < r^2 < 0.22$, summary **Table 4.4.1** and **Figure 4.4.3**). On the other hand, non-parametric analysis indicated that seven training behavior variables displayed a monotonic relationship to at least one of the three test Pre-CS freezing measurements (see **Tables S6-8** in **Supplementary data**). These Spearman correlations, however, generally did not follow readily identifiable linear or non-linear functions, as the plots reveal (**Figure S1** in **Supplementary data**), except for the duration of Pre rearing, which followed a weak linear relationship (**Figure 4.4.3C**). Thus, there seems to be little potential in any single measure of the training behavior to overall predict the observed fear generalization upon re-exposure to the familiar testing chamber.

Cluster analysis reveals a high-anxiety profile predictive of fear generalization

Although correlation analysis failed to detect suitable predictors for generalization of fear to a known context for our experimental protocol, we explored whether considering all training phases (Pre, TS1, ITI, TS2 and Post) simultaneously for each animal, might reveal a training behavior profile predictive of Pre-CS freezing during test. To address this possibility,

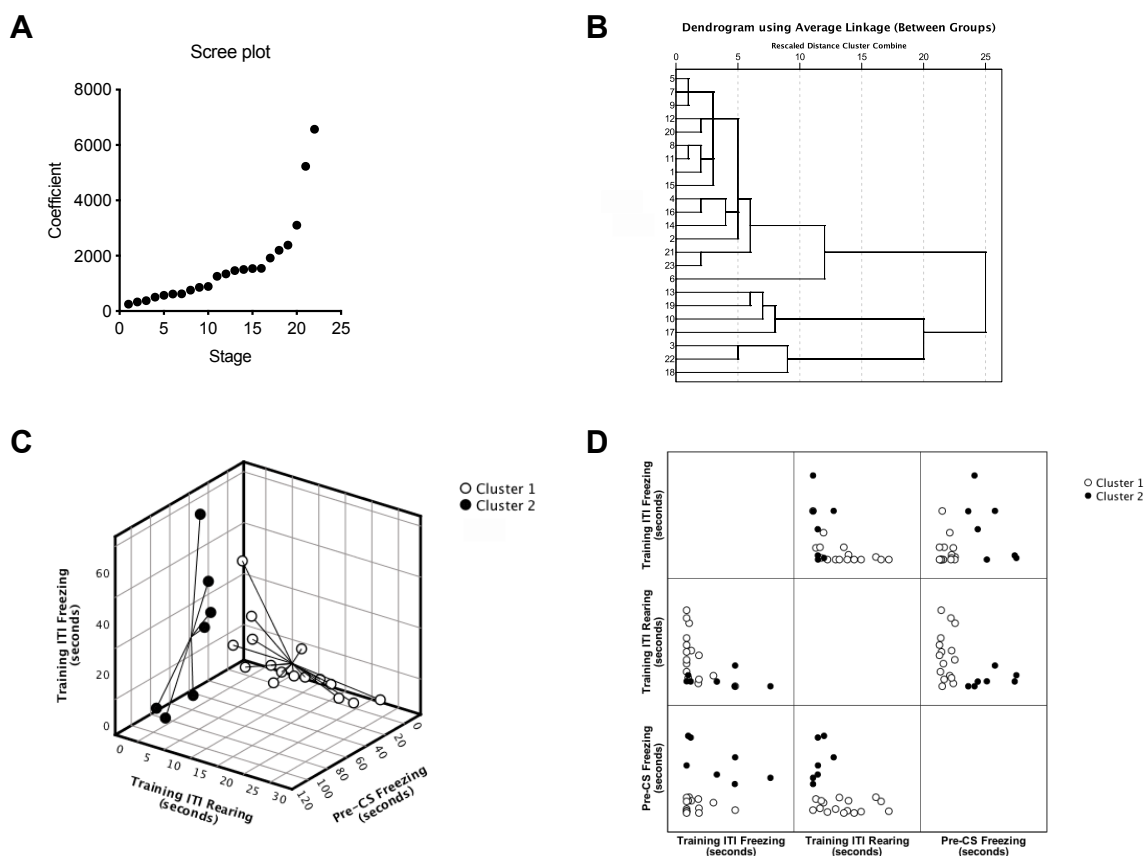
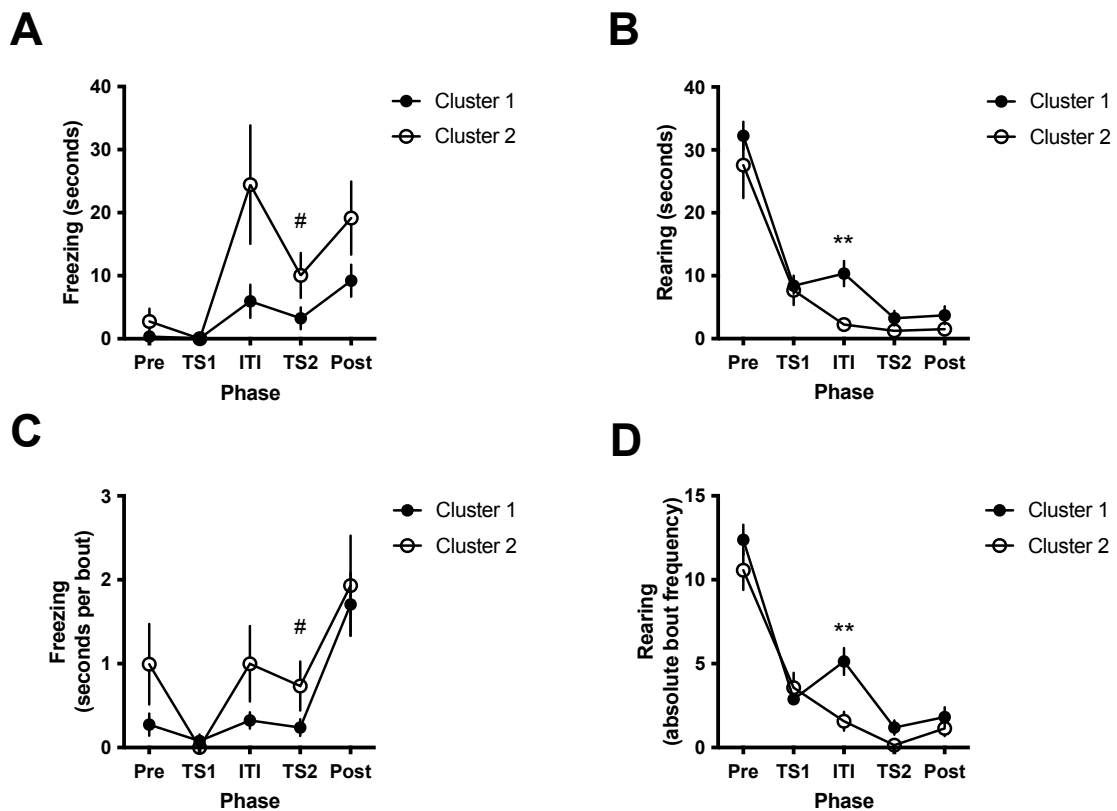


Figure 4.4.4. Hierarchical cluster analysis produces two major clusters of animals. We analyzed dependent variables Freezing, Rearing and Grooming duration (in seconds) for all training phases (Pre, TS1, ITI, TS2, Post), and Freezing duration for both testing phases (Pre-CS, CS) for each rat. Here we show a scree plot (**A**) taken from the agglomeration schedule (Table S5) and a clustering dendrogram (**B**) indicating at least two meaningful clusters. Freezing and rearing during the Training ITI are the best predictors for Pre-CS freezing during the test, and are thus displayed labeled by cluster, as a 3D scatter plot with cluster centroid spikes (**C**). Providing more detail, these data also are presented as a 2D matrix plot (**D**).

we performed a hierarchical cluster analysis (detailed in the **Materials and methods** section). Proximity matrix and agglomeration schedule cross-analysis (see supplementary **Table S9** and **Table S10**), together with visual inspection of a coefficient scree plot (**Figure 4.4.4A**) and the clustering dendrogram (**Figure 4.4.4B**), pointed to two major clusters, one with size $n = 16$ (Cluster 1, C1) and one with size $n = 7$ (Cluster 2, C2) (**Figure 4.4.4C-D**). Given the group split, we reanalyzed normality of training parameters for each new set,

which has now doubled from 41 to 82 parameters. Because the D'Agostino & Pearson omnibus K^2 test is only valid for $20 \leq n \leq 200$ (D'Agostino & Pearson, 1973) (before clustering, $n = 23$), we assessed normality of C1 and C2 data using the Shapiro-Wilk W statistic, which can accommodate smaller sample sizes ($3 \leq n \leq 50$) (D'Agostino & Pearson, 1973). We found that only 39 parameters, 12 from C1 (~14.6%) and 27 from C2 (~32.9%) were normally distributed (see supplementary **Tables S11-14**), still leaving out a large set that is not (~52.4%). Ideally a non-parametric two-way mixed ANOVA-like analysis based on these two Clusters as between-subjects factor (C1 vs C2), and training Phase (Pre vs TS1 vs ITI vs TS2 vs Post) as the repeated factor should be carried out. However, nonparametric procedures with more than one factor are not generally acceptable (Zar, 2013), and Friedman's test is only suited for a single repeated factor. We therefore decided to use two-sample t -tests (comparing C1 vs C2) for pairs of normally distributed samples, and non-parametric Mann-Whitney U -tests (comparing C1 vs C2) for pairs where at least one variable was not normally distributed (see supplementary **Table S15**). There were significant differences between C1 and C2 for only 8 (19.5%) of the 41 parameters. Student's t -tests revealed significant differences between the clusters for ITI rearing duration ($t(19.81) = 3.664$, $p = 0.002$) and absolute bout number ($t(20.78) = 3.583$, $p = 0.002$) during training, as well as for CS freezing absolute bout number ($t(17.67) = 5.277$, $p < 0.001$) during testing; Mann-Whitney U -tests detected significant differences between the clusters for TS2 freezing duration ($U =$



(See caption on next page.)

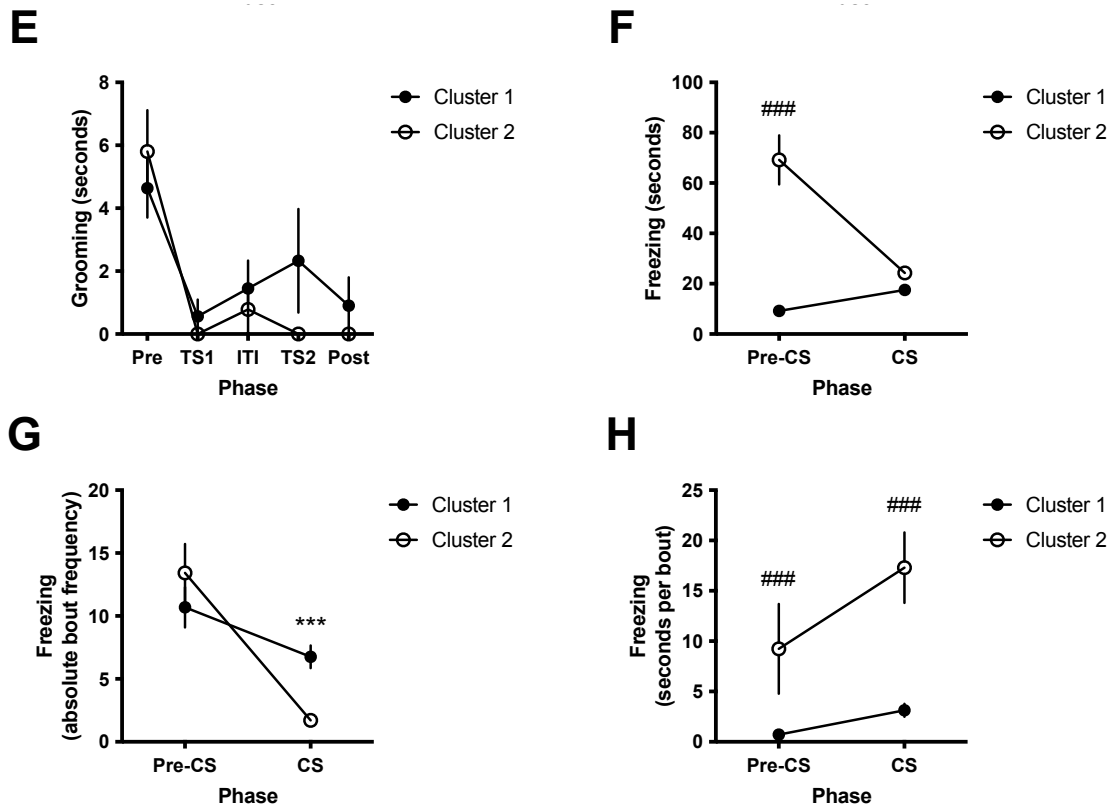


Figure 4.4.5. High freezing and low rearing after first shock presentation predict fear generalization during test. Displayed are behavioral profiles of animals in Clusters 1 and 2 for time (in seconds) spent freezing (A), rearing (B) and grooming (E), as well as average bout duration (in seconds per bout) of freezing (C) and absolute bout frequency of rearing (D) during training. We also show freezing duration (F), absolute bout frequency (G) and average bout duration (H) during test. We performed two-sample hypothesis testing using Student's *t* test on parameters with normal distribution, and Mann-Whitney *U*-test on non-normal data (see supplementary **Table S15**). Data points represent mean $\pm$ 1 SEM; Student's *t*-test: ** $p < 0.01$, *** $p < 0.001$; Mann-Whitney *U*-test: # $p < 0.05$, ### $p < 0.001$.

23.0, $z = -2.336$, $p = 0.019$) and average bout duration ($U = 26.0$, $z = -2.123$, $p = 0.034$) during training, as well as for Pre-CS freezing duration ($U = 0.0$, $z = -3.743$, $p < 0.001$), and Pre-CS ($U = 0.0$, $z = -3.743$, $p < 0.001$) and CS ($U = 2.0$, $z = -3.608$, $p < 0.001$) freezing average bout duration during testing. Most importantly, the two clusters differed numerically in freezing and rearing following the first shock during training (during ITI and TS2 phases; **Figure 4.4.5**), with Cluster 1 displaying low freezing / high rearing, and Cluster 2 displaying high freezing / low rearing, as predictors of low and high Pre-CS freezing, respectively. These data suggest that fear generalization to the testing context (Context A) is best predicted by the number of freezing and rearing bouts, their summed durations and average bout durations occurring after the first CS-US pairing, that is during the intertrial interval (ITI) and/or second tone presentation (TS2) of the training session in Context B. However, this relationship is neither linear nor exclusive given the correlation analysis data above,

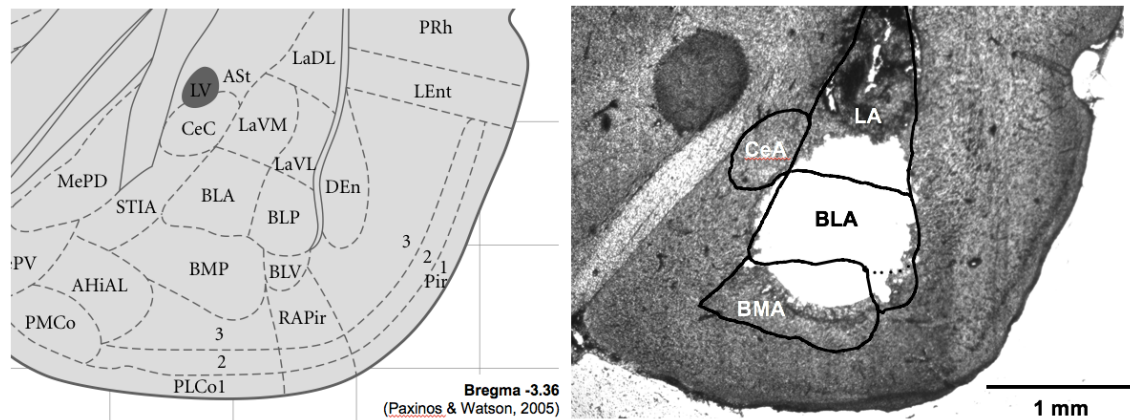


Figure 4.4.6. Amygdala punches for BL tissue collection. In order to analyze the expression of AMPARs at amygdala synapses, we collected amygdala tissue from selected animals with a 1-mm hollow needle directed at the basolateral (BL¹) amygdala nuclei. We here show a representative microscopy image of a rat brain slice after punching, illustrating that most of the tissue was collected from the BL and lateral (La¹) amygdala nuclei, while sparing other nuclei including the central (Ce¹) and the basomedial (BM¹) nuclei.

¹Note: BLA, LA, CeA and BM, respectively, in the right image.

indicating a behavioral bifurcation (mostly high freezing / low rearing vs mostly low freezing / high rearing) instead of a continuous, intermixed freezing/rearing gradient.

Pre-CS freezing positively correlates with GluA1 expression at amygdala synapses

We tested whether the observed differences in individual Pre-CS freezing levels could be explained by variations in native populations of AMPARs in the BL nucleus (**Figure 4.4.6**) and therefore measured their expression in post-synaptic densities (PSDs) and extra-synaptic regions (ESRs). We randomly selected six animals ($n = 6$) and correlated optical density measurements from the immunoblots directly with Pre-CS freezing levels during test in Context B. This revealed a highly significant covariation of freezing with synaptically expressed GluA1 (sGluA1, $r^2 = 0.9648$, $p < 0.001$), but not extra-synaptically expressed GluA1, although we still detected a strong trend (eGluA1, $r^2 = 0.6217$, $p = 0.0624$). There were no significant differences for expression of either synaptic (sGluA2) or extra-synaptic GluA2 (eGluA2) (**Figure 4.4.7**). *Post-hoc* power analysis (Pearson Product-Moment tool in SPSS) resulted in high statistical power (0.995) for a sample size $n = 6$ and a Pearson correlation parameter $r = 0.9822$. These data indicate that the expressed levels of GluA1 in spines of the BL nucleus relate to the degree of fear generalization observed in an animal.

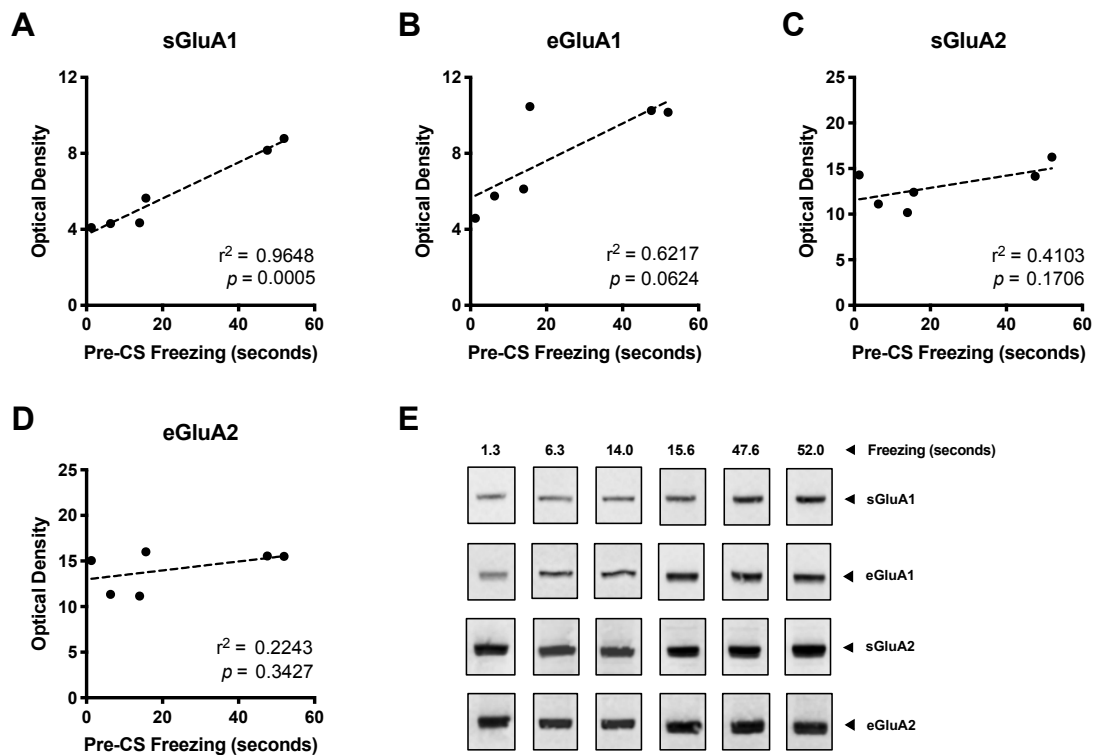


Figure 4.4.7. Synaptic GluA1 expression at the BL nucleus correlates with fear generalization during testing. Differential centrifugation of BL tissue taken from selected animals displaying varying degrees of testing Pre-CS freezing yielded synaptic and extra-synaptic fractions containing PSD proteins. Quantification of these fractions via optical densitometry revealed dissimilar expression of synaptic (sGluA1, sGluA2) (**A**, **C**) and extra-synaptic (eGluA1, eGluA2) (**B**, **D**) AMPARs, with GluA1 showing the highest degree of linear correlation with Pre-CS freezing duration (in seconds) during test. Squared Pearson coefficient (r^2) and respective p-value are indicated as scatter plot insets. Blots of all proteins analyzed on each fraction are shown (**E**) with Pre-CS freezing levels displayed as phase percentage above the blots.

4.5 Supplementary data

The following **Tables (S1-15)** and **Figure (S1)** were submitted as supplementary data along with the main research article.

Table S1. D’Agostino & Pearson omnibus K^2 normality test applied to the parameters of the acquisition session, according to **Figure 4.4.2A** (see main text for details). Test statistic (K^2) and p -value are indicated. Gray shading indicates a positive result for normality ($p > 0.05$). Fr., Freezing; Re., Rearing; Gr., Grooming; s, seconds; s/b, seconds per bout; b#, bout number.

ACQUISITION SESSION										
	Pre Fr. (s)	TS1 Fr. (s)	ITI Fr. (s)	TS2 Fr. (s)	Post Fr. (s)	Pre Fr. (b#)	TS1 Fr. (b#)	ITI Fr. (b#)	TS2 Fr. (b#)	Post Fr. (b#)
K^2	52.59	57.95	15.15	10.92	3.38	37.92	12.14	8.11	5.20	10.70
p -value	< 0.001	< 0.001	< 0.001	0.004	0.184	< 0.001	0.002	0.017	0.074	0.005
	Pre Fr. (s/b)	TS1 Fr. (s/b)	ITI Fr. (s/b)	TS2 Fr. (s/b)	Post Fr. (s/b)	Pre Re. (s)	TS1 Re. (s)	ITI Re. (s)	TS2 Re. (s)	Post Re. (s)
K^2	11.19	57.95	33.02	17.86	2.31	1.25	1.65	4.32	7.51	27.14
p -value	0.004	< 0.001	< 0.001	0.001	0.315	0.536	0.438	0.115	0.023	< 0.001
	Pre Re. (b#)	TS1 Re. (b#)	ITI Re. (b#)	TS2 Re. (b#)	Post Re. (b#)	Pre Re. (s/b)	TS1 Re. (s/b)	ITI Re. (s/b)	TS2 Re. (s/b)	Post Re. (s/b)
K^2	13.62	2.17	3.29	11.23	15.52	5.75	5.86	0.61	22.80	2.77
p -value	0.001	0.338	0.193	0.004	< 0.001	0.056	0.054	0.736	< 0.001	0.251
	Pre Gr. (s)	TS1 Gr. (s)	ITI Gr. (s)	TS2 Gr. (s)	Post Gr. (s)					
K^2	1.15	57.68	33.91	54.35	57.95					
p -value	0.562	< 0.001	< 0.001	< 0.001	< 0.001					

Table S2. D’Agostino & Pearson *omnibus* K^2 normality test applied to test session’s measured parameters, according to **Figure 4.4.2A** (see main text for details). Test statistic (K^2) and p -value are indicated. Gray shading indicates a positive result for normality ($p > 0.05$). Fr., Freezing; s, seconds; s/b, seconds per bout; b#, bout number.

	TEST SESSION					
	Pre-CS Fr. (s)	CS Fr. (s)	Pre-CS Fr. (b#)	CS Fr. (b#)	Pre-CS Fr. (s/b)	CS Fr. (s/b)
K^2	8.19	6.38	3.55	5.26	48.17	14.02
p -value	0.017	0.041	0.170	0.072	< 0.001	< 0.001

Table S3. Pearson correlation analysis for each acquisition and test parameter against 'Pre-CS Freezing (s)'. Pearson statistic (r) and p -value are indicated. Gray shading indicates significant correlations ($p < 0.05$). Fr., Freezing; Re., Rearing; Gr., Grooming; s, seconds; s/b, seconds per bout; b#, bout number.

CORRELATION ANALYSIS AGAINST 'Pre-CS Freezing (s)'										
	Pre Fr. (s)	TS1 Fr. (s)	ITI Fr. (s)	TS2 Fr. (s)	Post Fr. (s)	Pre Fr. (b#)	TS1 Fr. (b#)	ITI Fr. (b#)	TS2 Fr. (b#)	Post Fr. (b#)
r	0,085	-0,081	0,216	0,186	0,255	0,023	0,240	0,249	0,232	0,219
p -value	0,701	0,714	0,322	0,394	0,240	0,918	0,271	0,251	0,286	0,316
	Pre Fr. (s/b)	TS1 Fr. (s/b)	ITI Fr. (s/b)	TS2 Fr. (s/b)	Post Fr. (s/b)	Pre Re. (s)	TS1 Re. (s)	ITI Re. (s)	TS2 Re. (s)	Post Re. (s)
r	0,127	-0,081	0,210	0,244	0,050	-0,075	-0,144	-0,403	-0,103	-0,201
p -value	0,564	0,714	0,337	0,262	0,821	0,734	0,511	0,056	0,639	0,357
	Pre Re. (b#)	TS1 Re. (b#)	ITI Re. (b#)	TS2 Re. (b#)	Post Re. (b#)	Pre Re. (s/b)	TS1 Re. (s/b)	ITI Re. (s/b)	TS2 Re. (s/b)	Post Re. (s/b)
r	0,017	0,097	-0,367	-0,195	-0,068	-0,163	-0,356	-0,058	0,153	-0,148
p -value	0,938	0,661	0,085	0,374	0,757	0,457	0,096	0,794	0,486	0,499
	Pre Gr. (s)	TS1 Gr. (s)	ITI Gr. (s)	TS2 Gr. (s)	Post Gr. (s)	CS Fr. (s)	Pre-CS Fr. (b#)	CS Fr. (b#)	Pre-CS Fr. (s/b)	CS Fr. (s/b)
r	0,207	-0,174	-0,009	-0,232	-0,081	0,395	0,156	-0,572	0,769	0,797
p -value	0,342	0,428	0,966	0,287	0,714	0,062	0,476	0,004	0,000	0,000

Table S4. Pearson correlation analysis for each acquisition and test parameter against 'Pre-CS Freezing (b#)'. Pearson statistic (r) and p -value are indicated. Gray shading indicates significant correlations ($p < 0.05$).

CORRELATION ANALYSIS AGAINST 'Pre-CS Freezing (b#)'										
	Pre Fr. (s)	TS1 Fr. (s)	ITI Fr. (s)	TS2 Fr. (s)	Post Fr. (s)	Pre Fr. (b#)	TS1 Fr. (b#)	ITI Fr. (b#)	TS2 Fr. (b#)	Post Fr. (b#)
r	0,159	0,292	0,398	0,348	0,240	-0,021	0,257	0,237	0,262	0,175
p -value	0,468	0,177	0,060	0,104	0,269	0,924	0,237	0,276	0,228	0,426
	Pre Fr. (s/b)	TS1 Fr. (s/b)	ITI Fr. (s/b)	TS2 Fr. (s/b)	Post Fr. (s/b)	Pre Re. (s)	TS1 Re. (s)	ITI Re. (s)	TS2 Re. (s)	Post Re. (s)
r	0,382	0,292	0,416	0,420	0,240	-0,426	0,127	-0,163	-0,258	-0,358
p -value	0,072	0,177	0,048	0,046	0,271	0,043	0,563	0,459	0,234	0,094
	Pre Re. (b#)	TS1 Re. (b#)	ITI Re. (b#)	TS2 Re. (b#)	Post Re. (b#)	Pre Re. (s/b)	TS1 Re. (s/b)	ITI Re. (s/b)	TS2 Re. (s/b)	Post Re. (s/b)
r	-0,079	0,130	-0,138	0,102	-0,091	-0,394	-0,007	-0,347	-0,361	-0,470
p -value	0,719	0,553	0,531	0,643	0,681	0,063	0,973	0,105	0,090	0,024
	Pre Gr. (s)	TS1 Gr. (s)	ITI Gr. (s)	TS2 Gr. (s)	Post Gr. (s)	Pre-CS Fr. (s)	CS Fr. (s)	CS Fr. (b#)	Pre-CS Fr. (s/b)	CS Fr. (s/b)
r	0,406	-0,242	0,354	-0,197	0,292	0,156	0,324	-0,221	-0,238	0,156
p -value	0,055	0,266	0,097	0,367	0,177	0,476	0,132	0,311	0,274	0,477

Table S5. Pearson correlation analysis for each acquisition and test parameter against 'Pre-CS Freezing (s/b)'. Pearson statistic (r) and p -value are indicated. Gray shading indicates significant correlations ($p < 0.05$).

CORRELATION ANALYSIS AGAINST 'Pre-CS Freezing (s/b)'										
	Pre Fr. (s)	TS1 Fr. (s)	ITI Fr. (s)	TS2 Fr. (s)	Post Fr. (s)	Pre Fr. (b#)	TS1 Fr. (b#)	ITI Fr. (b#)	TS2 Fr. (b#)	Post Fr. (b#)
r	-0,048	-0,075	-0,081	-0,055	0,186	-0,078	0,074	0,117	0,108	0,165
p -value	0,828	0,734	0,715	0,802	0,395	0,723	0,736	0,595	0,624	0,452
	Pre Fr. (s/b)	TS1 Fr. (s/b)	ITI Fr. (s/b)	TS2 Fr. (s/b)	Post Fr. (s/b)	Pre Re. (s)	TS1 Re. (s)	ITI Re. (s)	TS2 Re. (s)	Post Re. (s)
r	-0,095	-0,075	-0,073	-0,018	0,046	0,173	-0,160	-0,234	-0,073	-0,035
p -value	0,668	0,734	0,740	0,934	0,837	0,430	0,466	0,283	0,740	0,873
	Pre Re. (b#)	TS1 Re. (b#)	ITI Re. (b#)	TS2 Re. (b#)	Post Re. (b#)	Pre Re. (s/b)	TS1 Re. (s/b)	ITI Re. (s/b)	TS2 Re. (s/b)	Post Re. (s/b)
r	0,165	0,059	-0,257	-0,155	0,019	-0,023	-0,258	0,278	0,084	0,117
p -value	0,452	0,791	0,236	0,481	0,930	0,917	0,235	0,199	0,703	0,595
	Pre Gr. (s)	TS1 Gr. (s)	ITI Gr. (s)	TS2 Gr. (s)	Post Gr. (s)	Pre-CS Fr. (s)	CS Fr. (s)	Pre-CS Fr. (b#)	CS Fr. (b#)	CS Fr. (s/b)
r	-0,053	-0,084	-0,091	-0,120	-0,075	0,769	0,316	-0,238	-0,372	0,710
p -value	0,810	0,702	0,679	0,584	0,734	0,000	0,142	0,274	0,081	0,000

Table S6. Spearman correlation analysis for each acquisition and test parameter against 'Pre-CS Freezing (s)'. Spearman statistic (r_s) and p -value are indicated. Gray shading indicates significant correlations ($p < 0.05$).

CORRELATION ANALYSIS AGAINST 'Pre-CS Freezing (s)'										
	Pre Fr. (s)	TS1 Fr. (s)	ITI Fr. (s)	TS2 Fr. (s)	Post Fr. (s)	Pre Fr. (b#)	TS1 Fr. (b#)	ITI Fr. (b#)	TS2 Fr. (b#)	Post Fr. (b#)
r_s	0,091	-0,032	0,370	0,429	0,310	-0,115	0,383	0,407	0,407	0,419
p -value	0,678	0,884	0,082	0,041	0,149	0,602	0,072	0,054	0,054	0,046
	Pre Fr. (s/b)	TS1 Fr. (s/b)	ITI Fr. (s/b)	TS2 Fr. (s/b)	Post Fr. (s/b)	Pre Re. (s)	TS1 Re. (s)	ITI Re. (s)	TS2 Re. (s)	Post Re. (s)
r_s	0,105	-0,032	0,310	0,394	0,213	-0,140	0,016	-0,460	-0,148	-0,193
p -value	0,633	0,884	0,150	0,063	0,330	0,524	0,941	0,027	0,500	0,377
	Pre Re. (b#)	TS1 Re. (b#)	ITI Re. (b#)	TS2 Re. (b#)	Post Re. (b#)	Pre Re. (s/b)	TS1 Re. (s/b)	ITI Re. (s/b)	TS2 Re. (s/b)	Post Re. (s/b)
r_s	0,102	0,198	-0,427	-0,141	-0,140	-0,271	-0,120	-0,266	-0,154	-0,282
p -value	0,642	0,366	0,042	0,522	0,525	0,210	0,587	0,220	0,484	0,192
	Pre Gr. (s)	TS1 Gr. (s)	ITI Gr. (s)	TS2 Gr. (s)	Post Gr. (s)	CS Fr. (s)	Pre-CS Fr. (b#)	CS Fr. (b#)	Pre-CS Fr. (s/b)	CS Fr. (s/b)
r_s	0,374	-0,103	0,128	-,469*	-0,032	0,356	0,509	-0,607	0,930	0,660
p -value	0,078	0,640	0,561	0,024	0,884	0,095	0,013	0,002	0,000	0,001

Table S7. Spearman correlation analysis for each acquisition and test parameter against 'Pre-CS Freezing (b#)'. Spearman statistic (r_s) and p -value are indicated. Gray shading indicates significant correlations ($p < 0.05$).

CORRELATION ANALYSIS AGAINST 'Pre-CS Freezing (b#)'										
	Pre Fr. (s)	TS1 Fr. (s)	ITI Fr. (s)	TS2 Fr. (s)	Post Fr. (s)	Pre Fr. (b#)	TS1 Fr. (b#)	ITI Fr. (b#)	TS2 Fr. (b#)	Post Fr. (b#)
r_s	0,241	0,306	0,398	0,332	0,257	-0,105	0,305	0,322	0,343	0,292
p -value	0,269	0,156	0,060	0,122	0,237	0,633	0,157	0,134	0,109	0,176
	Pre Fr. (s/b)	TS1 Fr. (s/b)	ITI Fr. (s/b)	TS2 Fr. (s/b)	Post Fr. (s/b)	Pre Re. (s)	TS1 Re. (s)	ITI Re. (s)	TS2 Re. (s)	Post Re. (s)
r_s	0,267	0,306	0,353	0,370	0,263	-0,416	0,077	-0,252	-0,174	-0,259
p -value	0,218	0,156	0,099	0,082	0,225	0,048	0,728	0,245	0,426	0,232
	Pre Re. (b#)	TS1 Re. (b#)	ITI Re. (b#)	TS2 Re. (b#)	Post Re. (b#)	Pre Re. (s/b)	TS1 Re. (s/b)	ITI Re. (s/b)	TS2 Re. (s/b)	Post Re. (s/b)
r_s	-0,153	0,070	-0,207	-0,019	-0,174	-0,354	0,090	-0,292	-0,236	-0,406
p -value	0,487	0,750	0,343	0,931	0,428	0,098	0,684	0,176	0,279	0,055
	Pre Gr. (s)	TS1 Gr. (s)	ITI Gr. (s)	TS2 Gr. (s)	Post Gr. (s)	Pre-CS Fr. (s)	CS Fr. (s)	CS Fr. (b#)	Pre-CS Fr. (s/b)	CS Fr. (s/b)
r_s	0,328	0,017	0,360	-0,183	0,306	0,509	0,337	-0,184	0,305	0,329
p -value	0,126	0,938	0,091	0,404	0,156	0,013	0,116	0,399	0,158	0,125

Table S8. Spearman correlation analysis for each acquisition and test parameter against 'Pre-CS Freezing (s/b)'. Spearman statistic (r_s) and p -value are indicated. Gray shading indicates significant correlations ($p < 0.05$).

CORRELATION ANALYSIS AGAINST 'Pre-CS Freezing (s/b)'										
	Pre Fr. (s)	TS1 Fr. (s)	ITI Fr. (s)	TS2 Fr. (s)	Post Fr. (s)	Pre Fr. (b#)	TS1 Fr. (b#)	ITI Fr. (b#)	TS2 Fr. (b#)	Post Fr. (b#)
r_s	0,082	-0,064	0,329	0,368	0,228	-0,119	0,383	0,348	0,322	0,315
p -value	0,709	0,771	0,125	0,084	0,296	0,588	0,072	0,104	0,135	0,143
	Pre Fr. (s/b)	TS1 Fr. (s/b)	ITI Fr. (s/b)	TS2 Fr. (s/b)	Post Fr. (s/b)	Pre Re. (s)	TS1 Re. (s)	ITI Re. (s)	TS2 Re. (s)	Post Re. (s)
r_s	0,093	-0,064	0,285	0,313	0,145	-0,030	0,043	-0,466	-0,074	-0,260
p -value	0,675	0,771	0,187	0,146	0,509	0,891	0,846	0,025	0,738	0,230
	Pre Re. (b#)	TS1 Re. (b#)	ITI Re. (b#)	TS2 Re. (b#)	Post Re. (b#)	Pre Re. (s/b)	TS1 Re. (s/b)	ITI Re. (s/b)	TS2 Re. (s/b)	Post Re. (s/b)
r_s	0,085	0,290	-0,399	-0,126	-0,196	-0,178	-0,126	-0,308	-0,076	-0,313
p -value	0,700	0,180	0,059	0,568	0,370	0,415	0,568	0,153	0,732	0,146
	Pre Gr. (s)	TS1 Gr. (s)	ITI Gr. (s)	TS2 Gr. (s)	Post Gr. (s)	Pre-CS Fr. (s)	CS Fr. (s)	Pre-CS Fr. (b#)	CS Fr. (b#)	CS Fr. (s/b)
r_s	0,291	-0,119	0,011	-0,535	-0,064	0,930	0,331	0,305	-0,485	0,572
p -value	0,178	0,588	0,960	0,009	0,771	0,000	0,123	0,158	0,019	0,004

Table S9. Proximity matrix for the hierarchical cluster of cases labeled by rat number. Analysis was run on IBM® SPSS® Statistics software with Squared Euclidean Distance as dissimilarity measure between rat variable matrices. Variables were freezing, rearing and grooming duration at all acquisition phases (Pre, TS1, ITI, TS2, Post) and freezing duration at both test phases (Pre-CS, CS) for each rat.

Squared Euclidean Distance																							
Case	1:1	2:2	3:3	4:4	5:5	6:6	7:7	8:8	9:9	10:10	11:11	12:12	13:13	14:14	15:15	16:16	17:17	18:18	19:19	20:20	21:21	22:22	23:23
1:1	0	1956	10806	1651	1241	3619	862	609	1039	8016	640	870	5657	1148	927	945	8854	4286	4595	1060	822	11484	1225
2:2	1956	0	8014	1351	1824	3013	1308	979	2157	5411	1178	1244	3172	1702	1249	1255	7190	3594	3443	1337	1300	8401	1722
3:3	10806	8014	0	9629	8360	12707	7178	8024	9436	2962	10029	10506	4884	11355	7163	11681	8842	2489	5817	8264	11459	1501	12163
4:4	1651	1351	9629	0	1805	2874	1420	1031	2276	5530	1589	1730	2908	1600	1495	571	6449	3346	4109	2026	2030	8061	2588
5:5	1241	1824	8360	1805	0	1548	251	872	276	4456	989	946	2689	1760	1051	1024	4319	3682	1410	1240	2042	9494	2381
6:6	3619	3013	12707	2874	1548	0	2383	2972	2337	5516	3135	3094	3295	3576	3795	1998	3229	7179	2652	4182	4073	12729	3964
7:7	862	1308	7178	1420	251	2383	0	310	379	4562	646	745	2745	1365	559	941	5504	2636	1890	909	1700	8150	1969
8:8	609	979	8024	1031	872	2972	310	0	1040	5573	374	879	3676	993	567	777	7231	2488	3184	1025	1107	8391	1249
9:9	1039	2157	9436	2276	276	2337	379	1040	0	6271	856	561	3757	1712	1067	1200	6198	4572	2317	1078	2006	11041	2319
10:10	8016	5411	2962	5530	4456	5516	4562	5573	6271	0	7408	7600	1535	8003	5484	6987	1995	2631	2302	6377	8467	2771	9131
11:11	640	1178	10029	1589	989	3135	646	374	856	7408	0	518	4709	804	784	808	8593	4014	3685	930	840	11068	979
12:12	870	1244	10506	1730	946	3094	745	879	561	7600	518	0	4643	941	812	737	8163	4904	3645	618	931	11558	1243
13:13	5657	3172	4884	2908	2689	3295	2745	3676	3757	1535	4709	4643	0	5335	3705	3834	2374	3245	1534	4500	6463	4543	7035
14:14	1148	1702	11355	1600	1760	3576	1365	993	1712	8003	804	941	5335	0	1543	909	8934	4801	4819	1580	1388	11524	1727
15:15	927	1249	7163	1495	1051	3795	559	567	1067	5484	784	812	3705	1543	0	1361	7377	2438	3046	738	1248	8096	1493
16:16	945	1255	11681	571	1024	1998	941	777	1200	6987	808	737	3834	909	1361	0	6788	4914	3708	1519	1343	11246	1709
17:17	8854	7190	8842	6449	4319	3229	5504	7231	6198	1995	8593	8163	2374	8934	7377	6788	0	7067	2231	8048	9863	8532	10338
18:18	4286	3594	2489	3346	3682	7179	2636	2488	4572	2631	4014	4904	3245	4801	2438	4914	7067	0	3909	3589	4903	2281	5291
19:19	4595	3443	5817	4109	1410	2652	1890	3184	2317	2302	3685	3645	1534	4819	3046	3708	2231	3909	0	3506	5404	7529	5757
20:20	1060	1337	8264	2026	1240	4182	909	1025	1078	6377	930	618	4500	1580	738	1519	8048	3589	3506	0	1003	9521	1762
21:21	822	1300	11459	2030	2042	4073	1700	1107	2006	8467	840	931	6463	1388	1248	1343	9863	4903	5404	1003	0	12046	504
22:22	11484	8401	1501	8061	9494	12729	8150	8391	11041	2771	11068	11558	4543	11524	8096	11246	8532	2281	7529	9521	12046	0	12704
23:23	1225	1722	12163	2588	2381	3964	1969	1249	2319	9131	979	1243	7035	1727	1493	1709	10338	5291	5757	1762	504	12704	0

Table S10. Agglomeration schedule for the hierarchical cluster of cases labeled by rat number, iterated using IBM® SPSS® Statistics software with Squared Euclidean Distance as dissimilarity measure between rat variable matrices.

Stage	Cluster Combined		Coefficients	Stage Cluster First Appears		Next Stage
	Cluster 1	Cluster 2		Cluster 1	Cluster 2	
1	5	7	250,825	0	0	2
2	5	9	327,628	1	0	10
3	8	11	374,108	0	0	7
4	21	23	504,468	0	0	16
5	4	16	570,638	0	0	11
6	12	20	618,287	0	0	9
7	1	8	624,273	0	3	8
8	1	15	759,705	7	0	9
9	1	12	854,092	8	6	10
10	1	5	889,501	9	2	12
11	4	14	1254,434	5	0	12
12	1	4	1340,111	10	11	13
13	1	2	1461,867	12	0	16
14	3	22	1500,821	0	0	19
15	13	19	1533,634	0	0	17
16	1	21	1543,2	13	4	20
17	10	13	1918,619	0	15	18
18	10	17	2199,96	17	0	21
19	3	18	2384,819	14	0	21
20	1	6	3104,126	16	0	22
21	3	10	5227,677	19	18	22
22	1	3	6572,195	20	21	0

Table S11. Shapiro-Wilk normality test applied to Cluster 1 (C1, n = 16) of the parameters measured during the acquisition. Test statistic (*W*) and *p*-value are indicated. Gray shading indicates a positive result for normality ($p > 0.05$). Fr., Freezing; Re., Rearing; Gr., Grooming; s, seconds; s/b, seconds per bout; b#, bout number.

ACQUISITION SESSION (C1)										
	Pre Fr. (s)	TS1 Fr. (s)	ITI Fr. (s)	TS2 Fr. (s)	Post Fr. (s)	Pre Fr. (b#)	TS1 Fr. (b#)	ITI Fr. (b#)	TS2 Fr. (b#)	Post Fr. (b#)
<i>W</i>	0.561	0.273	0.643	0.549	0.810	0.697	0.729	0.798	0.819	0.704
<i>p</i>-value	< 0.001	< 0.001	< 0.001	< 0.001	0.004	< 0.001	< 0.001	0.003	0.005	< 0.001
	Pre Fr. (s/b)	TS1 Fr. (s/b)	ITI Fr. (s/b)	TS2 Fr. (s/b)	Post Fr. (s/b)	Pre Re. (s)	TS1 Re. (s)	ITI Re. (s)	TS2 Re. (s)	Post Re. (s)
<i>W</i>	0.573	0.273	0.796	0.637	0.910	0.974	0.949	0.935	0.727	0.704
<i>p</i>-value	< 0.001	< 0.001	0.002	< 0.001	0.119	0.897	0.480	0.297	< 0.001	< 0.001
	Pre Re. (b#)	TS1 Re. (b#)	ITI Re. (b#)	TS2 Re. (b#)	Post Re. (b#)	Pre Re. (s/b)	TS1 Re. (s/b)	ITI Re. (s/b)	TS2 Re. (s/b)	Post Re. (s/b)
<i>W</i>	0.837	0.916	0.961	0.724	0.779	0.903	0.855	0.974	0.682	0.843
<i>p</i>-value	0.009	0.145	0.679	< 0.001	0.001	0.088	0.016	0.902	< 0.001	0.011
	Pre Gr. (s)	TS1 Gr. (s)	ITI Gr. (s)	TS2 Gr. (s)	Post Gr. (s)					
<i>W</i>	0.898	0.295	0.487	0.404	0.273					
<i>p</i>-value	0.074	< 0.001	< 0.001	< 0.001	< 0.001					

Table S12. Shapiro-Wilk normality test applied to Cluster 1 (C1, $n = 16$) of the parameters measured during the test session. Test statistic (W) and p -value are indicated. Gray shading indicates a positive result for normality ($p > 0.05$). Fr., Freezing; s, seconds; s/b, seconds per bout; b#, bout number.

TEST SESSION (C1)						
	Pre-CS Fr. (s)	CS Fr. (s)	Pre-CS Fr. (b#)	CS Fr. (b#)	Pre-CS Fr. (s/b)	CS Fr. (s/b)
W	0.858	0.875	0.949	0.936	0.949	0.827
p-value	0.018	0.033	0.470	0.306	0.470	0.006

Table S13. Shapiro-Wilk normality test applied to Cluster 2 (C2, $n = 7$) of the parameters measured during the acquisition session. Test statistic (W) and p -value are indicated. Gray shading indicates a positive result for normality ($p > 0.05$). Fr., Freezing; Re., Rearing; Gr., Grooming; s, seconds; s/b, seconds per bout; b#, bout number. ^(a)All values were zero, impossible to calculate W .

ACQUISITION SESSION (C2)										
	Pre Fr. (s)	TS1 Fr. (s)	ITI Fr. (s)	TS2 Fr. (s)	Post Fr. (s)	Pre Fr. (b#)	TS1 Fr. (b#)	ITI Fr. (b#)	TS2 Fr. (b#)	Post Fr. (b#)
W	0.602	(a)	0.889	0.913	0.940	0.638	0.937	0.962	0.969	0.958
p-value	< 0.001		0.268	0.417	0.636	< 0.001	0.615	0.833	0.894	0.800
	Pre Fr. (s/b)	TS1 Fr. (s/b)	ITI Fr. (s/b)	TS2 Fr. (s/b)	Post Fr. (s/b)	Pre Re. (s)	TS1 Re. (s)	ITI Re. (s)	TS2 Re. (s)	Post Re. (s)
W	0.751	(a)	0.793	0.848	0.956	0.926	0.961	0.841	0.453	0.853
p-value	0.013		0.035	0.118	0.784	0.518	0.825	0.102	< 0.001	0.130
	Pre Re. (b#)	TS1 Re. (b#)	ITI Re. (b#)	TS2 Re. (b#)	Post Re. (b#)	Pre Re. (s/b)	TS1 Re. (s/b)	ITI Re. (s/b)	TS2 Re. (s/b)	Post Re. (s/b)
W	0.955	0.969	0.915	0.453	0.859	0.929	0.962	0.894	0.453	0.832
p-value	0.771	0.889	0.432	< 0.001	0.147	0.546	0.834	0.296	< 0.001	0.084
	Pre Gr. (s)	TS1 Gr. (s)	ITI Gr. (s)	TS2 Gr. (s)	Post Gr. (s)					
W	0.908	(a)	0.453	(a)	(a)					
p-value	0.380		< 0.001							

Table S14. Shapiro-Wilk normality test applied to Cluster 2 (C2, $n = 7$) of the parameters measured during the test session. Test statistic (W) and p -value are indicated. Gray shading indicates a positive result for normality ($p > 0.05$). Fr., Freezing; s, seconds; s/b, seconds per bout; b#, bout number.

TEST SESSION (C2)						
	Pre-CS Fr. (s)	CS Fr. (s)	Pre-CS Fr. (b#)	CS Fr. (b#)	Pre-CS Fr. (s/b)	CS Fr. (s/b)
W	0.903	0.907	0.943	0.833	0.657	0.840
p-value	0.349	0.376	0.670	0.086	0.001	0.099

Table S15. Two-sample hypothesis testing applied to Cluster 1 (C1) vs Cluster 2 (C2) parameters. A Shapiro-Wilk normality test summary is shown (see **Tables S11-14** for details; N, normally distributed). Student's *t* test was applied when both C1 and C2 were normally distributed; otherwise, Mann-Whitney *U* test was chosen. Test statistic (*U* or *t*) and corresponding *p*-values are indicated. Gray shading indicates a significant difference was detected ($p < 0.05$). Fr., Freezing; Re., Rearing; Gr., Grooming; s, seconds; s/b, seconds per bout; b#, bout number. ^(a)*W* was not calculated.

Parameter	Shapiro-Wilk normality test		Two-Sample Hypothesis Test	Statistic		2-tailed <i>p</i> -value
	C1	C2		<i>U</i>	<i>t</i>	
Pre Fr. (s)			Mann-Whitney	40.0		0.189
TS1 Fr. (s)		(a)	Mann-Whitney	52.5		0.508
ITI Fr. (s)		N	Mann-Whitney	28.0		0.054
TS2 Fr. (s)		N	Mann-Whitney	23.0		0.019
Post Fr. (s)		N	Mann-Whitney	35.5		0.170
Pre Fr. (b#)			Mann-Whitney	45.0		0.409
TS1 Fr. (b#)		N	Mann-Whitney	28.0		0.059
ITI Fr. (b#)		N	Mann-Whitney	29.0		0.071
TS2 Fr. (b#)		N	Mann-Whitney	29.5		0.074
Post Fr. (b#)		N	Mann-Whitney	27.5		0.055
Pre Fr. (s/b)			Mann-Whitney	40.0		0.189
TS1 Fr. (s/b)		(a)	Mann-Whitney	52.5		0.508
ITI Fr. (s/b)			Mann-Whitney	31.0		0.085
TS2 Fr. (s/b)		N	Mann-Whitney	26.0		0.034
Post Fr. (s/b)	N	N	<i>t</i> test		-0.318	0.756
Pre Re. (s)	N	N	<i>t</i> test		0.819	0.436
TS1 Re. (s)	N	N	<i>t</i> test		0.275	0.789
ITI Re. (s)	N	N	<i>t</i> test		3.664	0.002
TS2 Re. (s)			Mann-Whitney	44.0		0.325
Post Re. (s)		N	Mann-Whitney	50.5		0.701
Pre Re. (b#)		N	Mann-Whitney	44.0		0.418
TS1 Re. (b#)	N	N	<i>t</i> test		-0.727	0.489
ITI Re. (b#)	N	N	<i>t</i> test		3.583	0.002
TS2 Re. (b#)			Mann-Whitney	40.0		0.189
Post Re. (b#)		N	Mann-Whitney	51.0		0.726
Pre Re. (s/b)	N	N	<i>t</i> test		0.462	0.652
TS1 Re. (s/b)		N	Mann-Whitney	37.0		0.204
ITI Re. (s/b)	N	N	<i>t</i> test		0.897	0.395
TS2 Re. (s/b)			Mann-Whitney	46.0		0.412
Post Re. (s/b)		N	Mann-Whitney	51.5		0.754
Pre Gr. (s)	N	N	<i>t</i> test		-0.722	0.484
TS1 Gr. (s)		(a)	Mann-Whitney	49.0		0.339
ITI Gr. (s)			Mann-Whitney	50.0		0.579
TS2 Gr. (s)		(a)	Mann-Whitney	38.5		0.105
Post Gr. (s)		(a)	Mann-Whitney	52.5		0.508
Pre-CS Fr. (s)		N	Mann-Whitney	0.0		< 0.001
CS Fr. (s)		N	Mann-Whitney	32.0		0.109
Pre-CS Fr. (b#)	N	N	<i>t</i> test		-0.974	0.349
CS Fr. (b#)	N	N	<i>t</i> test		5.277	< 0.001
Pre-CS Fr. (s/b)	N		Mann-Whitney	0.0		< 0.001
CS Fr. (s/b)		N	Mann-Whitney	2.0		< 0.001

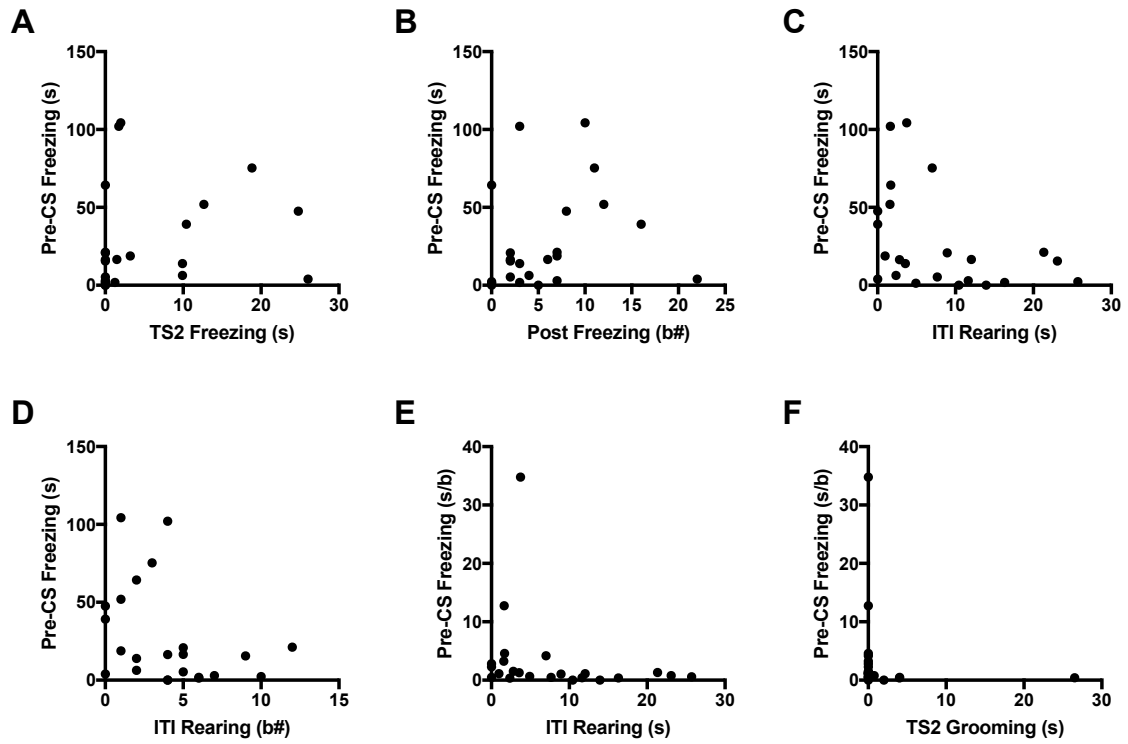


Figure S1. Non-parametric correlation analysis between test Pre-CS freezing duration and training TS2 freezing duration (**A**), Post freezing bout number (**B**), ITI Rearing duration (**C**) and ITI Rearing bout number (**D**), and between test Pre-CS duration per bout and training ITI Rearing duration (**E**) and TS2 Grooming duration (**F**) showed statistical significance ($p < 0.05$ in A-E, $p < 0.01$ in F; see Tables S6-8 and main text for details). As can be seen, however, these Spearman correlations are hard to ascribe to definite non-linear functions.

4.6 Discussion

We explored behavioral and synaptic markers in male rats that could account for individual differences in fear generalization to a testing context after auditory fear conditioning. Cluster analysis revealed that specific behavioral profiles observed during training predicted fear generalization. Specifically, we observed that animals jointly expressing high freezing and low rearing after the first tone-shock pairing presented also with high Pre-CS freezing during test, i.e., fear generalized to the testing context. Furthermore, post-synaptic GluA1-AMPA levels at BL nucleus' synapses measured shortly after the test correlated with Pre-CS freezing as well. These results suggest that learning during training may affect the extent to which animals generalize their auditory fear over time, depending on individual differences in glutamatergic drive in the BL nucleus.

Of all variables measured, rearing during training appeared as the most useful predictor for later fear generalization. Although relatively little is known about the exact functions and learning outcomes of rearing in rats, one of its accepted purposes seems to be the gathering of spatial or contextual information to map the present environment, notably when rats recognize environmental novelty (Lever et al., 2006). Rearing can be affected by fear and anxiety, which modulate this exploratory behavior in a non-linear fashion. One theoretical account argues that rats likely reduce rearing in novel environments when they experience low anxiety but very high fear; on the other hand, rearing will be readily expressed when some anxiety, or excitement, is present due to novelty but fear is low (Gray & McNaughton, 2000). In other words, the more rats experience a new situation as threatening, the less they will rear, and, consequently, the less spatial-contextual knowledge about the environment they likely will acquire. This may shed light on our finding that the less rats expressed rearing after they experienced the first shock, the more they generalized their freezing to a known context – these rats can be assumed to have explored the spatial environment less than others, and arrived thus at less detailed, less precise representations of the chamber in which they received foot shocks. When presented with the test context that somewhat resembled the original training context (e.g., due to similar materials and general dimensions, but also due to the relative similarity of the events compared to their daily lives in the colony), they may have falsely recognized this chamber as the one in which they received the foot shocks, thus expressing fear in the form of freezing in anticipation of a similar unpleasant experience. Rats also express other exploratory behaviors to encode the environment, such as sniffing, horizontal scanning and moving around, and this may explain why some of the animals that displayed low rearing during the inter-trial interval nevertheless showed relatively low generalized contextual fear (Fig. 4D). Regardless, our data strongly

suggest that rearing relates to context encoding in a standard AFC protocol, and future experimental studies will be required to explore this effect further.

Because auditory and contextual fear conditioning involve somewhat different pathways in the brain, the first being relatively independent of the hippocampus compared to the latter (Phillips & LeDoux, 1992; Zelikowsky et al., 2014), it is not surprising that presenting the CS prompted rats to freeze strongly during test, but less so to the context alone. Indeed, while variations in hippocampal spatial encoding can account for different Pre-CS freezing during test, plasticity in the amygdala also mediates the acquisition and expression of various emotional aspects of the overall fear memory (Zelikowsky et al., 2014). Although it has been shown that activity in the circuit connecting the anterior cingulate cortex (ACC) to both ventral (vHC) (Bian et al., 2019; Cullen et al., 2015) and dorsal hippocampus (dHC) (Nagayoshi et al., 2022) promotes fear memory generalization, the amygdala is critical for fear discrimination during learning, receiving afferent signals from, and relaying efferent signals to the auditory cortex as well as other brain structures, including the vHC (Bin Kim & Cho, 2017). In particular, ACC and vHC projections to the BL nucleus seem to control fear expressed in novel, nonthreatening environments, which is dependent upon training intensity (ACC) or time (vHP) (Ortiz et al., 2019). Disruptions of these pathways may thus also be involved in fear generalization phenomena. Other pathways, including an interaction of the bed nucleus of the stria terminalis (BNST) and the amygdala, underpin generalization responses upon fear learning (Duvarci et al., 2009; Sullivan et al., 2004), while the medial prefrontal cortex (mPFC) and the nucleus reuniens (NR) attenuate this effect by fine-tuning the discrimination between different contexts (Likhtik et al., 2014; Xu & Sudhof, 2013). Also, plasticity in the medial geniculate nucleus (MgN) of the thalamus, which inputs to the amygdala, seems to modulate encoding of fear memory discrimination (Ferrara et al., 2017). Moreover, the neural circuitry responsible for the execution of freezing involves amygdala central nuclei (Ce)-driven disinhibition of the ventrolateral periaqueductal gray (vIPAG), producing downstream characteristic body immobilization (Tovote et al., 2016).

Considering that the BL nucleus has extensive connections to the Ce nucleus, it is not surprising that individual modulation differences in BL nuclei activity produce opposite expression of freezing and rearing behaviors. This may partially explain our clustering data, which indicate that two distinct clusters with high freezing / low rearing during the intertrial intervals of the conditioning session predicted contextual fear generalization during the test session. Interestingly, during rearing episodes in mice, place cells in the HC stop firing, while a different subset of different pyramidal cells becomes activated (Barth et al., 2018). This might reflect that the animals are in the process of updating existing spatial representation. Because HC place cell firing patterns change following amygdala stimulation (Kim et al., 2012), the stability of spatial representation during context encoding may be

compromised when there is amygdala-dependent emotional distress, such as during AFC, possibly contributing to generalized fear, impairing the acquisition of comprehensive spatial representations of the environment.

Whether the amygdala directly or indirectly influences the HC is still a matter of debate, but these findings suggest a possible scenario explaining why increased fear generalization in terms of Pre-CS freezing correlated with less rearing during conditioning and higher GluA1 expression in BL neurons in our studies: possibly, for rats that reacted with a stronger fear response during auditory fear conditioning, activation in the amygdala was more pronounced, which, in turn, attenuated rearing (as discussed above) and diminished the accuracy of spatial representations of the conditioning context; this then can lead to higher generalized fear expression in future tests because similar contexts cannot be well distinguished from the conditioning context. In agreement with our hypothesis, S^{831}/S^{845} double phosphomutant mice, which display reduced GluA1-AMPA synaptic delivery and signaling, also show significantly more rearing in the open field compared with wild-type animals (Kiselycznyk et al., 2013).

Of the amygdala nuclei, the BL has been the one most extensively studied in the context of fear memory. About 80% of BL synapses are excitatory (Tovote et al., 2015), and one of the signature features of fear learning is increased expression of AMPARs at around one-third of LA synapses (Rumpel et al., 2005). AMPARs are heterotetramers composed of dimers of subunit (GluA1-4) dimers, but the GluA4 subunit is barely expressed in adult neurons (Henley & Wilkinson, 2016). In the amygdala of both mice and humans, the vast majority (> 98%) of GluA2 subunits undergo Q/R editing, such that AMPARs containing the edited version are calcium-impermeable (Paschen et al., 2002; Brande-Eilat et al., 2015). Conversely, when the edited GluA2 subunit is mostly absent, such as during plasticity when synaptic GluA1 increases, receptors become calcium-permeable (Clem & Huganir, 2010). Several lines of research indicate that GluA2-lacking AMPARs in BL interneurons mediate LTP expression and fear learning (Clem & Huganir, 2010; Mahanty & Sah, 1998; Polepalli et al., 2010). There is also direct evidence for low expression of the GluA2 subunit in rat BL spines (Gryder et al., 2005) and general retention of receptors containing this subunit in the cytoplasm, at least in thalamo-amygdala synapses (Radley et al., 2007). On the other hand, GluA1 is increased in rat BL synapses following stressor exposure (Hubert et al., 2014) and acquisition of auditory and contextual fear conditioning is impaired in $GluA1^{-/-}$ mice, with LTP at thalamic inputs to LA neurons being completely absent (Humeau et al., 2007), which is not the case for $GluA3^{-/-}$ mice.

These studies indicate that synapses in the amygdala display a different AMPAR subunit profile, with GluA1 having a more prominent role, when compared to other brain areas, such as the hippocampus, where GluA2 takes the lead (Lu et al., 2009). It may therefore not be

surprising that we observed that freezing to the test context positively correlated with the amount of GluA1 expressed at post-synaptic densities (PSDs) of BL synapses, implying an increased preponderance of calcium-permeable AMPAR-related effects, namely via interneuron activation. Nevertheless, having sacrificed rats within 5 minutes following CS presentation during the test session, mobility of receptors in synaptic membranes following memory retrieval-induced synaptic activity needs to be considered when interpreting this correlation (Rao-Ruiz et al., 2011). Constitutively, in order just to maintain existing synaptic levels of AMPAR expression, it takes around 10 min for ~30% of AMPARs to exchange between synaptic and extra-synaptic membranes (Kerr & Blanpied, 2012). Although their diffusion rate is increased when animals are fear conditioned (Yu et al., 2008), removal of AMPARs from thalamo-amygdala synapses, similar to what occurs during LTD, or following fear memory retrieval (Bhattacharya et al., 2017; Nabavi et al., 2014; Rich et al., 2019), as is the case in our study, normally requires 15 minutes of low frequency (1 Hz) stimulation (Wang & Gean, 1999; Huang & Kandel, 2007). Thus, in the context of our 5-minute time window during which we removed animals from the testing chamber and sacrificed them, any changes in AMPAR content at amygdala PSDs should be minimal.

Using the anesthetic isoflurane before extracting the brain could also have affected synaptic expression of AMPARs. However, findings from studies focusing on the hippocampus, one of the brain areas most vulnerable to the effects of anesthetic substances (Kang et al., 2017; Deng et al., 2014), suggest that it is very unlikely that isoflurane could have produced quick changes in GluA1-AMPA numbers in the BL nucleus. For example, isoflurane exposure did not affect expression of GluA1 protein (Qu et al., 2013) or its mRNA levels (Uchimoto et al., 2014) in rat hippocampal lysates or synaptoneurosomes up to two hours after exposure. Furthermore, application of isoflurane in hippocampal neuronal cultures promoted more than a ~30% reduction in the excitatory post-synaptic current amplitude evoked by glutamate (100 μ M), but only for the highest tested concentration (1.22 μ M) (de Sousa et al., 2000). Taken together, these studies indicate that a post-synaptic mechanism for anesthesia action on glutamatergic synapses mostly involves receptor antagonization rather than receptor removal from the plasma membrane. Therefore, in our setting, very brief (< 2 min) isoflurane anesthesia before brain collection probably had no meaningful impact on AMPAR expression at amygdala isolated PSDs and therefore unlikely explains our findings.

It remains an open question why some animals reacted stronger than others to the first tone-shock pairing during auditory fear conditioning. One possible source of interindividual variation may reflect different influences of regulatory pathways, such as the serotonergic system, which tonically inhibits the amygdala (Asan et al., 2013). Several studies have found a direct relationship between inhibition of serotonin production and GluA1 expression

(Tran & Keele, 2011; Tran et al., 2013), making it possible that animals freezing strongly to the Pre-CS also present with decreased serotonergic signaling. On the other hand, since dendritic hypertrophy with increased number of spines has been found in rat BL nucleus' neurons following suboptimal maternal care (Guadagno et al., 2018), differential early-life stress may epigenetically contribute to these variances in fear generalization. Interestingly, excessive maternal licking and grooming leads to poorer threat discrimination upon AFC that directly results from decreased activity in the vHC (Nguyen et al., 2018). Projections from the vHC to the BL nucleus seem to regulate fear expressed in safe environments (Ortiz et al., 2019), and so it is possible that too much maternal care is responsible for vHC-driven inactivation of inhibitory routes within the BL nuclei. Also, increases in synaptic GluA1 have been found at least 6 days following a repeated, unpredictable foot shock protocol (Hubert et al., 2014), again implying a role for previous stressors in differential GluA1-AMPA expression. Because the amygdala receives inputs from diverse structures, including the vHC, BNST, mPFC, NR and MgN, and is under neuromodulatory control from several systems, proper fear discrimination will likely rely on adequate weighing and integration of various signals and on epigenetic changes within regional neural networks. Whether these or other mechanisms underlie the relationship between BL nucleus glutamate receptor expression and fear generalization will require careful future work.

In summary, we identified a high-freezing/low-rearing behavioral profile expressed during the first exposure to a CS-US pairing in a novel training context that predicts later fear generalization to a known test context. We also showed that the extent of fear generalization correlated with post-synaptic expression of GluA1-AMPA in the BL nucleus. Therefore, careful analysis of various behaviors expressed during training will provide information useful in explaining interindividual differences in fear expression and its neurobiological correlates in these widely used experimental protocols. Our work further confirms the involvement of BL nucleus post-synaptic GluA1-AMPA expression in the complex process of generalized fear expression, with implications for fear- and anxiety-related disorders, such as posttraumatic stress disorder (PTSD).

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5 Appendix

Where I allowed myself to digress more freely about some aspects of the brain and its neurons, namely their biophysics and classification systems.

5.1 A room with a view

Whatever the world is made of, in its intricate communication between a large zoo of some fundamental (and others not-so-fundamental) particles, using but four force carriers as currency according to the standard model (Pokorski, 2024), plus gravity, matter aggregates and gains identity in many ways, some seemingly unorganized heterogeneous mixtures — as the rocks that constitute the planets —, others more visibly organized and with some degree of voluntary motility — as the living organisms that populate the Earth, who knows if other worlds. Complexity, at all levels, arises out of simplicity by way of small unnoticeable steps along a great deal of time, and in the most noble and fascinating way: without any orchestrator, only through unstoppable interaction and continuous transformation of all parts. Unsurprisingly, this gives rise to many redundancies, as natural experimentation often finds different routes to the same ends: think of the PKM ζ protein story — for sure selected as the main atypical kinase isoform to perform its function, but quickly traded for by PKC ι/λ in mouse PKM ζ -knockouts (but see (Wang et al., 2016)). Nature indeed has properly provisioned. There is, of course, an evolutionary history of brains, as along the 3.5-4 Ga²⁸ or so that living forms exist (Schopf et al., 2018) many such natural experiments have been performed and, together with geological change, have allowed the dynamical selection of features most adequate for each organism to wander, reproduce and survive.

It is not an easy task to assure how much of neuronal mass can be considered a *brain*, as size and number are not necessarily its trademarks — and research has shown that how neurons organize, interconnect, and speak to each other seems to be more important (see work in connectomics) than their numbers. Still, there is a population continuum: from the single-cell protozoan *Paramecium* (frequently equated to a “swimming neuron”), through the 10³-neuron jellyfish, the 10⁵-neuron *Drosophila* (Greenspan, 2007), up to the 86.1×10⁹ neurons of the human brain (Azevedo et al., 2009). As each neuron is capable of thousands of synaptic contacts, one can only imagine the immensity of computational power carried

²⁸ Ga is a geological unit meaning giga-annum, that is, 10⁹ (one *thousand million*) years. In the present thesis we adopt the European long-scale naming system, as opposed to the USA short-scale system in which one giga-annum is one *billion* years.

out by such a network. But can each single synaptic contact code any aspect of an organism's behavioral repertoire, or even a *bit* of information from the surrounding environment? Does it even make sense to impart such weight on a single unit, which is itself part of something bigger? Indeed, each neuronal synapse by itself was never considered to bring much significance to behavior interpretation: as fascinating as the postsynaptic density may be, it is the whole synaptic and dendritic chorus, plus the assembly of the network, that ultimately are expected to allow for stimuli integration and motor response (i.e., behaviors). But the paradigm is changing — as we shall see ahead, evidence is accumulating at least for the importance of single neuron dendritic integration in modulating behaviors (Poirazi & Papoutsi, 2020). One should also pay close attention to which species, and most importantly which brain regions, are taken to model certain neuronal functions. Although reasonable to do during the second half of the 20th century, when relatively sparse and scattered brain

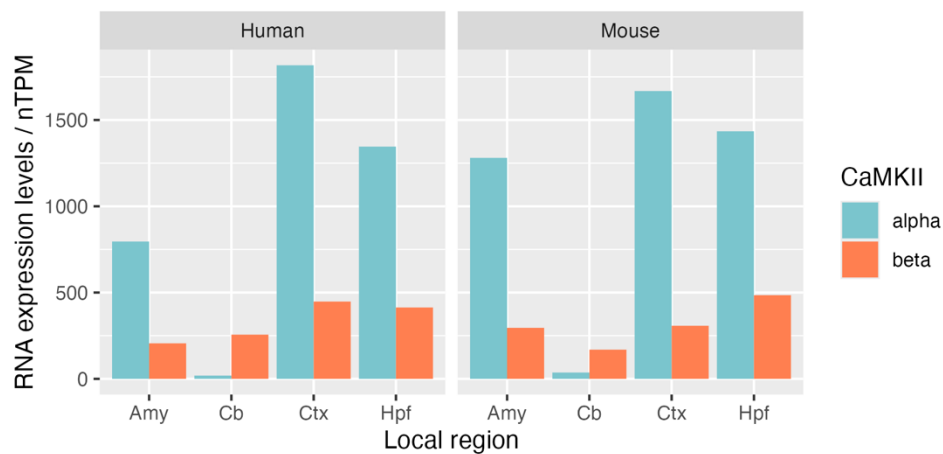


Figure 5.1.1. Comparison of RNA expression levels (nTPM, normalized transcripts per million) of CaMKII alpha and beta isoforms shown for four brain local regions in humans and mice. Amy, amygdala; Cb, cerebellum; Ctx, cerebral cortex; Hp, hippocampal formation. Data from the Human Protein Atlas (proteintlas.org).

research data was still available and all the little bits of the general puzzle were useful, it is currently useless to just state in broad terms which mechanisms are behind certain nervous phenomena, as very detailed region- and species-specific knowledge is already at hand. Even as recently as a decade and a half ago, for instance, CaMKII's function was discussed without regard to brain local region, such as the hippocampus or the cerebellum. CaMKII has four isotypes encoded in the human and mouse genomes (α , β , γ , and δ), two of the most studied and amply shown to be essential for learning and memory processes being CaMKII-alpha and CaMKII-beta. While both show similar inter-species patterns of isotype expression, clearly the hippocampus and the cerebellum, for example, do not agree on which isotype they prefer (**Figure 5.1.1**). As it so happens, the alpha and beta isotypes serve different synaptic functions, the former regulating functional activity and strength, the

latter responsible for dendritic morphology and number of synapses (Fink et al., 2003; Thiagarajan et al., 2002), such that their stoichiometric ratios heavily influence synaptic dynamics during learning and memory formation. This means that sentences like “the function of CaMKII in neurons is” are somewhat hollow unless one details the species, the brain region, and the isotype being considered, if not other relevant aspects such as the cell type (pyramidal neuron, inhibitory interneuron, etc.), the age of the animal, its stimulation history, and so on. As redundant as the proteins participating in them, learning and memory biochemical (and electrical) mechanisms also present numerous nuances depending on which organism one has under the amplifying lens. For instance, considering biochemical events at excitatory spines leading to vertebrate long-term potentiation (LTP) and invertebrate long-term facilitation (LTF), which came ultimately to be generally accepted as supporting long-term memory (LTM) processes in these subphyla, while persistent protein kinase A (PKA) activity seems important for LTF maintenance in marine seasnail *Aplysia* sensory neuron synapses (Müller & Carew, 1998), it is only transiently activated in mammalian hippocampal CA1 neurons during LTP induction (Tang & Yasuda, 2017). So, while both *Aplysia* and the mouse (and, of course, humans) display LTM phenomena, nature has certainly provided them with different tools to achieve it, such that a truly universal mechanism is probably a utopia. This is not a surprise given the different behavioral repertoires and habitat adaptations of these organisms: maybe LTM in the *Aplysia* is not as temporally long and involved as in mice and humans, which have different forms of LTM with different temporal and organizational characteristics. Also, their life spans are quite different (*A. californica*, e.g., only

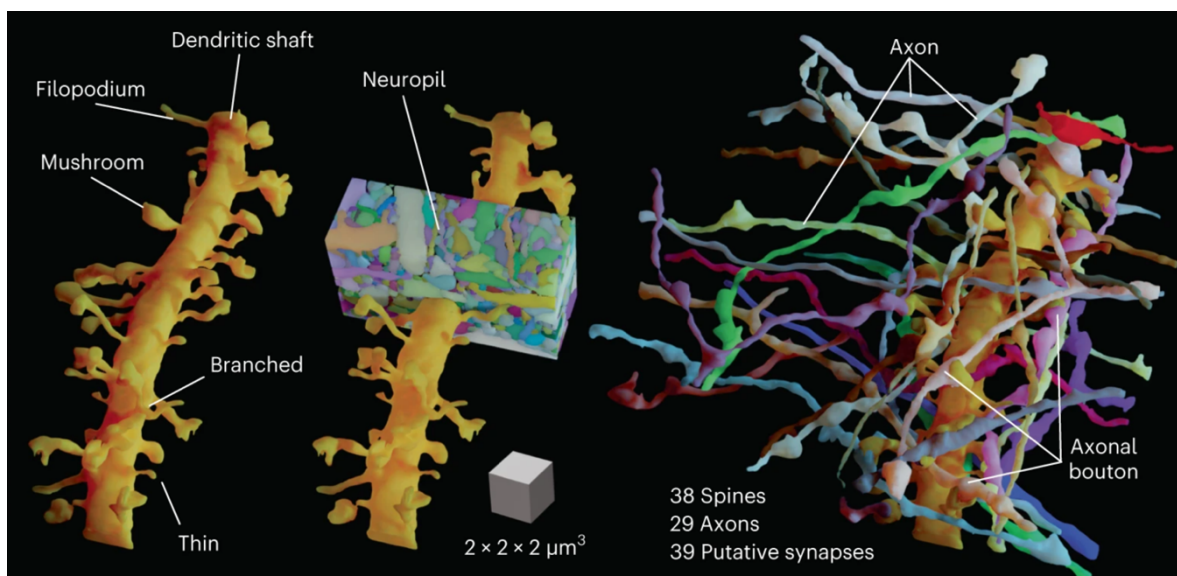


Figure 5.1.2. 3D reconstruction of a spiny dendrite in organotypic hippocampal slice cultures from a *Prox1-cre::Ai95* mouse. The authors used an integrated optical/machine-learning technology called LIONESS (live information-optimized nanoscopy enabling saturated segmentation). Reproduced from Fig. 3c in Velicky et al. (2023).

lives for about 1 year (Gerdes & Fieber, 2006)), so it is hard to imagine the biological need for mechanisms dedicated to expansive LTMs.

The nervous tissue is often a messy place (Velicky et al., 2023) (**Figure 5.1.2**). A great

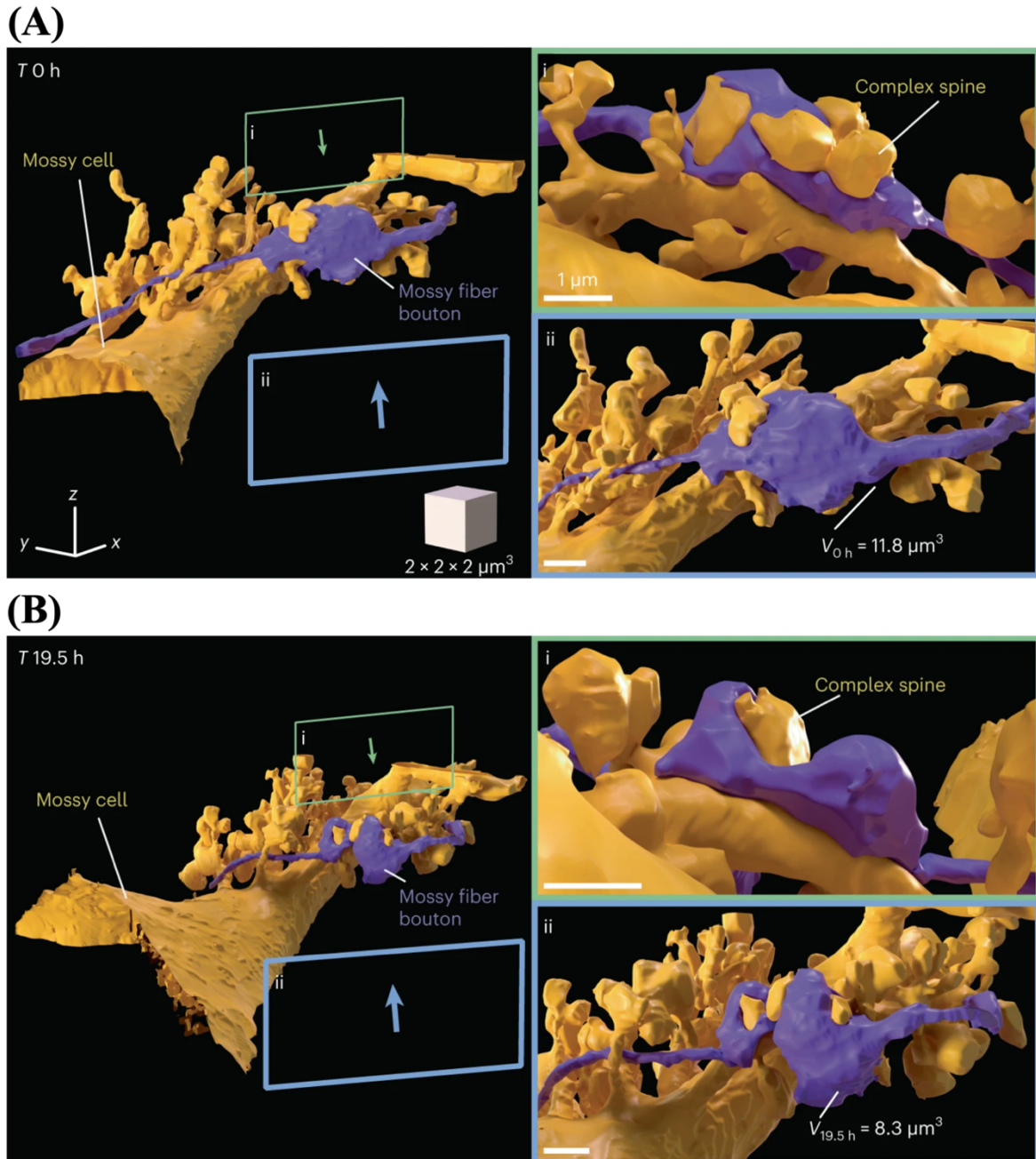


Figure 5.1.3. 3D reconstructions of a mossy fiber bouton (purple) and its postsynaptic partner, a hilar mossy cell (gold) with complex spines. The images were collected at time points (A) 0 h, and (B) 19.5 h following chemically induced activation. Reproduced from Fig. 5b in Velicky et al. (2023).

deal of the nervous mass is guided by a fit-where-you-can rule, in which specific circuit routes accommodate against each other while filling in the available space. This is not to say neuron positioning within an area, or synaptic contacts between neurons, are random

— several developmental factors make sure they are not, by carefully guiding, e.g., glutamatergic and GABAergic neurons and their connections according to a well-established functional plan (Hatanaka & Hirata, 2020; Schmitz et al., 2022). Neurons intermingle in the accessible space, and highly dynamically compress and relieve their connecting protrusions, acquiring the strangest shapes and turns. In fact, within a couple of hours, local microcircuits can dramatically change their structural aspect (**Figure 5.1.3**), which is not to say synaptic contacts also change, but certainly dendritic and axonal twists and turns do, perhaps to allow some connections to endure, while pruning others.

It is a wonder how much organized knowledge through careful experimentation has come out of such seemingly chaotic sceneries. I have often asked myself how is it that the electrochemical signals running along neuronal processes and originated in synaptic and action potentials' inputs avoid *parallel* influences from adjacent membranes, given the dense state of packed neural matter. Or do they? In other words, how is it that if you have two apposed dendrites and receive a synaptic signal on both independently, one does not influence the other? This is a difficult subject to come across in the connectionism-oriented literature, so even before trying to find the “needle in the haystack”, I made the following thought experiment some time ago which I wish to share with the reader: consider the simpler case of a single stationary point charge (ion), q , on one side of the plasma membrane. The magnitude of the voltage, $V(r)$, which is the work that can be done per unit charge by q on a test point, is given by $V(r) = \frac{U}{q_{test}} = -k \frac{q}{r}$, and since $k = 8.99 \times 10^9 \text{ N m}^2 \text{ C}^{-2}$, and $q = e = 1.6 \times 10^{-19} \text{ C}$, we know $kq \approx 1.44 \times 10^{-9} \text{ N m}^2 \text{ C}^{-1}$, and we have

$$V(r) = -\frac{1.44 \times 10^{-9}}{r}. \quad (1)$$

This is a steady-state scenario, so time is not included. Now, a typical membrane has thickness $r \approx 4 \times 10^{-9} \text{ m}$ (4 nm), and so, using equation (1), we get $V = -1.44/4 = -360 \text{ mV}$ (the minus sign is just a convention: the charges have opposite polarity, and so are attracted to each other). This means that an electrode placed 4 nm away from our ion would theoretically register an “attraction” voltage of approximately -360 mV. With two juxtaposed membranes ($\approx 8 \text{ nm}$) we would still have $V = -1.44/8 = -180 \text{ mV}$ of potential influence between a charge in one dendrite and a charge on another. For the voltage to drop considerably, say to something below 1 mV, our ion would have to be at least $1.44 \text{ }\mu\text{m}$ away, which is the whole diameter of a typical dendrite (Tian et al., 2022), and still a good approximation to physiological conditions (although average dendrite-to-dendrite distance is hard to estimate, a recent study found spine lengths ranged from $0.54 \text{ }\mu\text{m}$ to $3.96 \text{ }\mu\text{m}$ (average

$1.77 \mu\text{m} \pm 0.69 \mu\text{m}$), which may be considered an indirect estimate of inter-dendrite spacing) (Velicky et al., 2023). Certainly, we are simplifying our system to a single point charge, whereas the actual setup involves a distributed charge framework with many different contributing ions and different neuronal geometries. Also, given biological membranes are crowded with macromolecules, the actual interactions between charged ions on either side of them certainly occur over longer distances than just the membrane's average length, and the overall charge on both sides of the membrane may also not follow such simple numbers. But still, this simple exercise calculation reveals that there must be some inter-neurite membrane potential influence. This influence, I have learned from the “haystack”, has indeed been recognized in the 1940s (but has tortured neurophysiologists further back), and has been given a name, *ephaptic coupling* (Arvanitaki, 1942; Katz & Schmitt, 1940), to distinguish it from canonical *synaptic transmission* (which involves physical-chemical release of neurotransmitter *quanta*). Experiments with nervous tissue at the time were mostly conducted on peripheral nerves. Also, the influence of synaptic signals is quite strong, as opposed to the weak fields generated by ephaptic coupling. So it took another over 60 years for experimental confirmation of central, hippocampal neurons' sensitivity to external weak electric fields (Francis et al., 2003), and almost another further decade to effectively prove that endogenous brain activity influences neural function through weak field effects between adjacent neuronal processes, on the order of 0.5 mV, with fields under 5 mV/mm (Anastassiou et al., 2011) (just for comparison, when a single *synaptic* excitatory postsynaptic potential (EPSP), ~10 mV, considering only passive conduction, transverses the dendrite and arrives at the soma, it generates a *somatic* EPSP of the same order as that of the ephaptic weak fields, ~0.5 mV). Ephaptic and synaptic coupling are, thus, sister communication routes for neurons. We are just at the beginning of understanding this phenomenon, but it is becoming so important that in recent years there has already been an effort to accommodate this effect into dynamic models of neural activity (Aspart et al., 2016; Isakovic et al., 2018), and very recent research has shown that ephaptic coupling plays a significant role in synchronizing Purkinje cell firing in the cerebellum (Han et al., 2018), and in the propagation of the slow hippocampal periodic activity, thought to be related to memory consolidation during sleep (Chiang et al., 2019). If the reader thinks attentively about this, some of the effects of ephaptic coupling phenomena were already at hand: electroencephalography wouldn't be possible without it (how else would you detect anything at the scalp?), and neither would the *extracellular* recordings in the rabbit hippocampus that gave birth to the notion of LTP (Bliss & Lomo, 1973), given they were obtained *outside* neurons. Signals propagate more efficiently and more orderly along biological membranes (and within cell processes), but they still spread around electromagnetically into the available space to influence any chargeable particles, such as ions and proteins. Hence, the extracellular space

sort of *collects* from the dendritic spiny and synaptic quorum: partly because of ephaptic coupling, partly because when there is neural activity (ion exchanges through the membrane) the cell exterior is also affected by the removal and addition of positive and/or negative charges. Indeed, the field EPSP deflection mirrors the summated intracellular EPSPs along the time axis: they just operate at very different scales — while a *single* spine or dendritic EPSP takes on the order of several millivolts (mV), the field EPSP generated extracellularly from *many* stimulated synaptic contacts is a residual signal on the order of just a few microvolts (μ V).

5.2 A struggle of classes

In general, the CNS can be thought of as constituted by an arrangement of *local regions* or functional *centers* connected by pathways, or tracts (the most common route for signal travel), columns and laminae (within the cortex) and topographical maps (representing the spatial and sensory organization of the environment into adjacent neuronal regions in the cortex). The local regions (which we commonly call “brain areas”, e.g., the hippocampus, the amygdala, or the thalamus) are made up of interacting *dendritic subunits* (e.g., each hippocampal area [CA1, CA3, ...], or each amygdala [Ba, La, ...] or thalamic [LGN, MGN, ...] nuclei), apiece a set of *microcircuits*. Finally, microcircuits are triads of *input fibers* (axons or dendrites), *principal* or *relay neurons*, and *interneurons* (a type of *intrinsic neuron*, the other two being sensory and motor neurons), all interconnected via a plethora of *synapses* (Shepherd, 2004). Historically, synapses have been divided in two major types, simply named type 1 and type 2, first described in cortical axo-somatic and axo-dendritic contacts by George Gray in 1959 (Gray, 1959), but identified in many subcortical regions thereof. Synapses can occur between almost any two of the three canonical neuronal segments (soma, axon, and dendrites), including other non-neuronal structures (e.g., the neuromuscular junction), but the vast majority of them are axo-dendritic (G. Stuart et al., 2016). *Type 1* synapses, which have an asymmetrical distribution of densities when viewed via electron microscopy, have been found to mostly correlate with glutamatergic, excitatory transmission, and can be primarily found in dendritic spines and shafts, representing around 80-90% of a local region’s synapses. *Type 2* synapses, which are symmetrical in micrographic aspect, are GABAergic, have an inhibitory nature, and are mostly found in dendrite shafts (both proximal and distal), the neuronal soma, and the axon initial segment (AIS). Indeed, GABAergic interneurons constitute the most morphologically and functionally diverse cells within the forebrain, allowing for computational power gain over cortical microcircuits (Kepecs & Fishell, 2014). Comprising 10-20% of cortical neurons in rodents, almost all cortical GABAergic interneurons fall within three marker-expressing categories: ~40%

are parvalbumin (PV) interneurons, which mostly synapse to dendritic spines and distal shafts, and are fast-spiking; ~30% are somatostatin (STT) interneurons, with synapses onto proximal dendrites, the soma, and the AIS, and display regular- and burst-firing; the remaining ~30% are 5HT3aR-expressing, include VIP+ and VIP- interneurons, and preferentially synapse into the AIS (B. Rudy et al., 2011).

A major problem in biology is that there rarely is a single classification system that both humans and Nature agree upon. One might think that since neurons are *cells* there easily emerges a common sorting scheme for them, such as one based on their different shapes/morphologies or functions. Still, even neurons with similar shapes do differ in function. Electrophysiological experiments reveal that the same stimulus given to two adjacent excitatory neurons produces bursting activity in one of them while a single delayed spike on the other, which may occur because their dendritic trees are different in size, or connectivity, or even that their dendritic channel identities vary considerably — some displaying scaling, others less so. These disparities abound. One ought to think it useless to keep trying to find a classifying scheme, but this would render thinking and talking about these matters impractical, and of course although things are never so easy to overcome, they are not so difficult either. Despite their imperfection, classification systems are still very useful.

The morphological approach was pioneered by Santiago Ramón y Cajal (Ramón y Cajal, 1899, 1904) and Camillo Golgi (Golgi, 1886) as one of the first attempts to classify neurons, but has been slowly losing favor to functional classifications. Indeed, gene expression, epigenetic modification, firing history and patterning, etc., contrary to morphology, seem modifiable properties extremely dependent on developmental stage and plasticity events, i.e., a neuron's *history*. During development, for instance, the intracellular concentration of chloride is higher than outside some soon-to-be inhibitory interneurons, which means the Nernst equilibrium potential of chloride is reversed (compared to that of mature neurons) and the GABA neurotransmitter actually *excites* these cells (Ganguly et al., 2001). The functional identity of these neurons therefore changes over time (they start off excitable and end up being inhibitable) and is thus dependent on developmental stage and relative ion concentrations. Further, it is sometimes difficult to decide, neurochemically, which function a cell takes on a network: are dopamine-producing neurons excitatory or inhibitory? Some argue they are actually *both* (hence the preferred nomenclature *modulatory*), given the resulting effect of dopamine release is not dependent on the producing neuron (because dopamine is just... dopamine) but on *which* metabotropic dopamine receptors are expressed on the *postsynaptic* cell: D1-like receptors (D1 and D5) couple to G_s proteins (which *stimulate* adenylyl cyclase to produce excitatory effects via cAMP); D2-like receptors (D2 through D4) couple to $G_{i/o}$ proteins (which *inhibit* adenylyl cyclase, thus decreasing cAMP) (Beaulieu & Gainetdinov, 2011). Choosing which ID to assign to a dopamine-producing presynaptic

neuron is thus also dependent on which postsynaptic cell it delivers its neurotransmitter to. Take three neurons in succession ($A \rightarrow B \rightarrow C$: the dopamine-producing presynaptic neuron [A], and a postsynaptic GABA-producing neuron [B], which is presynaptic to yet a third postsynaptic cell [C]). If neuron B's synapses express D1-like receptors, presynaptic dopamine release by A will contribute to neuron B's excitation and make it release GABA, which will in turn inhibit the postsynaptic neuron C (if no other stimulation sources exist) — here, excitation produces downstream inhibition. On the other hand, if neuron B's synapses express D2-like receptors, that neuron will be inhibited by binding dopamine released by A, and either seize its inhibitory effect (no more GABA release) or remain silent (or further subjected to shunting inhibition²⁹ to other excitatory sources), which will ultimately lead to postsynaptic heightening of the excitation level of neuron C — excitation leads to, indirectly, possible excitation. This thought example already illustrates a rather complicated scenario with only three neurons connected in series; but what if, ultimately, dopamine release by neuron A leads to a certain behavior being simulated or inactivated, such as limb movement? While some neurons may function as “signaling bridges” along a pathway (e.g., a neuron which always excites the next one but has no direct part in behavioral decision), others may play more decisive roles for the organism. How far should we go on the network to decide on the ultimate effect of (in)activating a particular neuron? You can appreciate the complexity of this matter as we find that overall increases and decreases in neurotransmitter release may also not tell us the whole firing story (hence, functional significance) of a particular neuron. On the contrary — coming back to Ramón y Cajal and Golgi —, neuronal overall structure and shape, even accommodating connectivity dynamics (with a few exceptions), seem to change significantly less over time and space (except, perhaps, during some developmental and plasticity stages, as well as disease). Also, as we have already seen, the shape and complexity of dendritic arborizations indeed define part of a neuron's function. So, in a way, despite the physiological *Spiel*, there is always a common *Gestalt* to a certain class of neurons. But while it is true that out of structure one may find function on many instances — take, as an example, multipolar neurons, which display rich dendritic trees, making it no wonder these cells are responsible for a wealth (indeed the majority) of the brain's computations, both excitatory and inhibitory, which would probably not be possible if most neurons were, for instance, unipolar —, the opposite is equally true. i.e., that

²⁹ Shunting inhibition occurs when the excitability of a postsynaptic neuron has been decreased *before* it receives, e.g., an excitatory signal: this may occur, for instance, if the postsynaptic neuron first receives an IPSP from another source, making it lower its local potential below the resting state, such that the incoming EPSP is counteracted by the ongoing IPSP, and an experimenter sees no response from the neuron to the induced EPSP.

sometimes a particular function slowly *induces* the selection of a particular structure if, in a Darwinian spirit, it confers some adaptive advantage to the organism and, by extension, the population. Maybe function, being the purpose of structure, helps build the latter, and thus is intricately connected to it, lending identity to cells. Evolutionarily speaking, it makes sense to assume that every time a certain structure arose that didn't have an energetically meaningful function for its organism, it either vanished or was later adapted to serve another function. Maybe it is this morpho-functional “dance” which defines what a neuron is, not ever but *dynamically*.

The CNS, consisting of the brain and spinal cord, albeit a complex network of neurons, is nevertheless tangible. Neurons, asymmetric in nature, are themselves asymmetrically positioned to allow brains to control the body's interaction with its surroundings. Such is the case, for instance, of sensory and motor systems, which unidirectionally conduct signals towards or away from the brain, respectively. Functional neuroanatomy is already an old field, with many researchers contributing different nomenclatures. Following a somewhat painful and not ever very elucidative literature search (Kandel et al., 2021; Kepecs & Fishell, 2014; Masland, 2004; Panula & Nuutinen, 2013; Zeng & Sanes, 2017), I have tentatively, and very broadly, categorized neurons in the CNS into five main categories (see **Figure 5.2.1**): (a) **primary function**, which splits neurons into those that bring outside information into the CNS (*sensory*), those which conduct information out of the CNS, to muscles and glands (*motor*), and those that help perform calculations between sensory and motor neurons (*interneurons*), or between interneurons themselves; (b) **general morphology**, which is probably the broadest (and oldest) classification form, including *unipolar* (not found in vertebrates, and absent from **Figure 5.2.1**), *pseudo-unipolar*, *bipolar*, and *multipolar* shapes; (c) **connectivity**, which simply states if a neuron is carrying long-range signals towards a region (*afferent*), long-range signals away from a region (*efferent*, if very long-range, *projection*, or *relay* interneurons, if less longer-range), or within small, short-range circuits (*local*, sometimes called *intrinsic* interneurons); (d) **neurochemistry**, which, as we partly tackled above, distinguishes those neurons contributing to direct EPSP increases (*excitatory*), decreases (*inhibitory*), or both (*modulatory*); and finally (e) **specific morphology**, which pertains to the neuron's dendritic tree diversity and cell body shape (as visually inspected by researchers over the decades, and usually of multipolar neurons), giving rise to a classification into characteristic shapes such as *stellate* cells, *chandelier* cells, and *pyramidal* cells. Thus, e.g. CA3-to-CA1 neurons in the hippocampus (“CA3 neurons”) are (b) multipolar (c) projection (d) excitatory (e) pyramidal (a) interneurons. Now, the astute reader will immediately call for a mistake: these are *not* interneurons — s/he might say. But sure, they are. If classification systems are to be of any use, they must etymologically subserve their own meaning, and the prefix *inter-* means *between*, which in the context of the

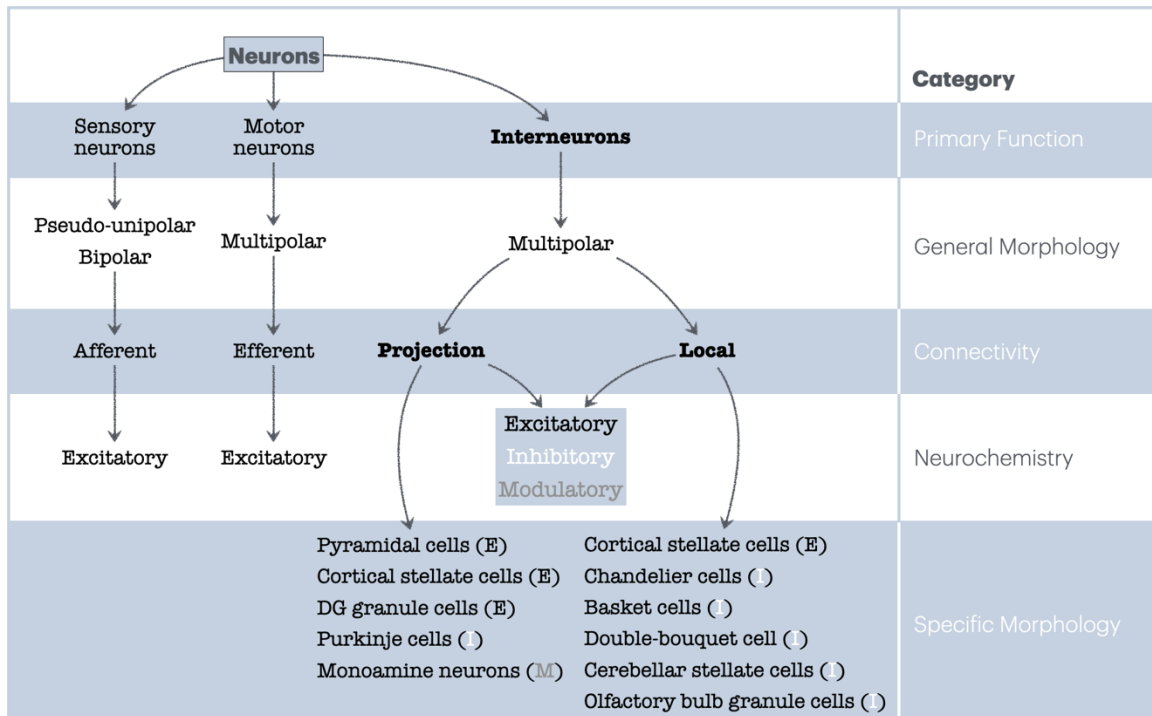


Figure 5.2.1. General scheme depicting five possible main categories of CNS neuron classification. The scheme is tentatively broad and assumed incomplete, given some exceptions can always be found for each category and research is constantly ongoing. Monoamine neurons include dopaminergic, serotonergic, (nor)ad-
renergic, and histaminergic cells. Note that while most projection interneurons are excitatory, local interneurons are mainly inhibitory. DG, dentate gyrus (hippocampus).

word ‘interneuron’ makes for *between neurons*. The pyramidal cells we just exemplified connect the CA3 to the CA1 region in the hippocampus, and so they exert their function *between* neurons of either dendritic subunit. They are not called pyramidal because they are excitatory, but because of the shape of their cell bodies (specific morphology), as much as interneurons are not obligatory inhibitory, they just modulate information between other neurons. And because their axons are rather long, pyramidal cells are *projection* neurons which, again, does not implicate excitation: Purkinje cells in the cerebellum, for example, although GABAergic (therefore inhibitory), are also projection neurons. It just so happens that most projection neurons in the CNS are indeed excitatory, and a large proportion of CNS interneurons are inhibitory (**Figure 5.2.1**), and from here stems the adapted wording laziness within the neuroscience field. But all neurons that are neither sensory nor motor in function, connect and modulate other neurons: and are thus interneurons.

Given connectivity and neurochemistry, as we have seen, may be postsynaptic-dependent categories, they frequently take the role of neuron-specific categorizers, allowing for more detailed neuron distinctions. Based on the above classification scheme, and hinting on the number and type of their extended processes (neurites), which are the axon and dendrites, we may expand on some of the alluded classes: *bipolar neurons* have two

distinctive processes, one axon and one dendrite, and (as *pseudo-unipolar neurons*, which start as bipolar and make up the majority of sensory neurons) are most common in *sensory systems*, found in such tissues as the retina and olfactory epithelium; *multipolar neurons*, on the other hand, have a single axon and can be classified based on the structure and function of their multiple dendrites. Thus, e.g., as mentioned above, *pyramidal* or *pyramidal-like neurons* have a triangular cell body and a patterned dendritic tree, including a basal, and a prominent apical dendrite, being found in the cerebral cortex, hippocampus, and, as we discussed in this thesis, the **amygdala**. *Purkinje cells* have a large, flat cell body and a densely branched dendritic tree, and can be found in the cerebellum. *Granule cells* have small cell bodies (resembling small granules) and a few short dendrites and are mostly found in many areas of the cerebrum. *Stellate cells* have a star-shaped appearance due to their multiple dendrites extending in various directions from the cellular soma, and are found in various brain regions, also including the cerebral cortex. Note that both granule and stellate cells can be excitatory *and* inhibitory (**Figure 5.2.1**). *Basket cells* are named for their basket-like aspect, having a dense network of axons that surround the cell bodies of other neurons, and are crucial for inhibitory functions. *Horizontal cells* are found in the retina, have long, horizontally aligned axons and play an important role in lateral inhibition. *Chandelier cells* have a distinctive candelabra-like appearance with terminal axonal branches that form vertical columns around the initial segments of other neurons, playing a crucial role in neuronal inhibition. *Double bouquet cells*, found in the cortex and olfactory bulb, have two bundles of dendrites resembling a flower bouquet, and are involved in odor processing. The list could go on (Paxinos, 2014) but already pays tribute to the vastness of the morphological landscape of central neurons. Though not included in the classification scheme of **Figure 5.2.1**, the morphological presence or absence of *spines* throughout dendritic trees is sometimes also used to characterize neuron types. While **spiny neurons** are characterized by the existence of spread-out dendritic spines, common in the cerebral cortex and several subcortical structured regions, being both excitatory and inhibitory (e.g. pyramidal and Purkinje cells, respectively), **aspinous neurons** lack dendritic spines and are typically inhibitory in nature (e.g. some types of stellate cells). Still, on average, about 75% of neocortical neurons possess spines on their dendritic protrusions (Koch & Zador, 1993). The **BL** nucleus of the amygdala closely follows these figures: around 10-20% of BL neurons are non-pyramidal cells (mostly inhibitory interneurons), and most of these have aspinous or spine-sparse dendrites, whilst the remainder majority are pyramidal-like, spiny neurons (McDonald, 2024).

It is important to recognize that while the pyramidal shape will tell the microscopist s/he is not looking at a Purkinje cell, this *per se* would have little meaning if function is not considered. Although e.g. pyramidal-shaped neurons are most commonly glutamate-

producing, and hence excitatory cells, stellate neurons may both be excitatory or inhibitory, even on the same local region (Needham & Paolini, 2006), so that finding a star-shaped neuron does not automatically define it neurochemically. Also, releasing excitatory or inhibitory neurotransmitters is only part of a cell's character: neurons produce many types of neurotransmitters, thousands of proteins, including many *ionic channels* which heavily influence, if not anything else, the *firing properties* of other neurons, as well as themselves. The last few decades have witnessed the entry of genetics and molecular biology into the scene, with many proposing “molecular barcodes” defining the “ground state”³⁰ of neurons via collections of gene expression profiles, correlating (or not) with particular firing patterns (Fishell & Heintz, 2013; Nelson et al., 2006; Poulin et al., 2016; Sugino et al., 2006). Many argue that the original fate of neurons is determined during development (there is a good recent review on amygdala development in rodents (Aerts & Seuntjens, 2021)), and that despite modification history, the molecular “ground state” should prevail in defining a neuron's identity. But taken too seriously, this approach ends with *somatic mosaicism* fostered by continuous somatic mutations, which originate single-cell genomic diversity (ergo, there are no two cells alike) (McConnell et al., 2017), and if there ever was a dream of neuronal classification, well, then no more. The reader may appreciate that, again, it seems the morpho-functional “dance” works best in the middle: too much functional and genetic discrimination leads to loss of a more general insight, while reliance on morphology only lends us a too foggy scenario. There should be some sort of compromise.

There are other morpho-functional considerations, maybe more in line with, or contiguous to our thinking, which still account for differences in, at least, ion channel expression. Indeed, a simple genetic barcode is arguably a poor descriptor for a highly dynamical cell: subcellular location is essential within neurons. Let's say we know a particular neuron

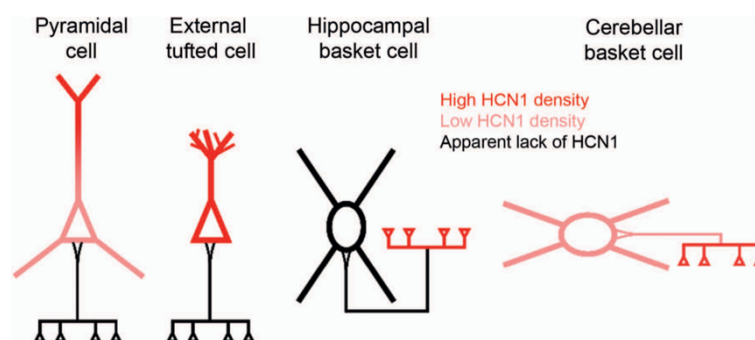


Figure 5.2.2. Subcellular, dendritic and axonic distribution of HCN1 in different CNS cell types. Reproduced from Stuart et al. (2016).

³⁰ This is a reference to excitation or energy levels of electrons in atoms: the ground state is the level of least energy. Some authors use this to signify a neuron's genetic “ground state”, i.e., the gene expression profile making up the basal ID of the cell. Anything that changes this “barcode” (e.g. disease) changes the cell's identity.

expresses the HCN1 subunit of hyperpolarization-activated cyclic nucleotide-gated (HCN) channels. Most CNS neurons express HCNs, given their role in setting the resting membrane potential; but both *dendritic* and axonal HCN subcellular expression patterns may be quite dissimilar for different neurons, with important integrative and computational consequences (**Figure 5.2.2**). (Notomi & Shigemoto, 2004; G. Stuart et al., 2016). The figure illustrates that cortical pyramidal cells display an HCN1 increasing proximal-to-distal gradient, meaning prolonged synaptic inhibition at dendritic distal sites will heighten the membrane potential there, while having less of an effect at proximal sites, or at somatic membranes. Hippocampal basket cells, on the other hand, have no HCN-dependent modulated integration at their dendrites. Hence, an alliance between morphology and ion channel

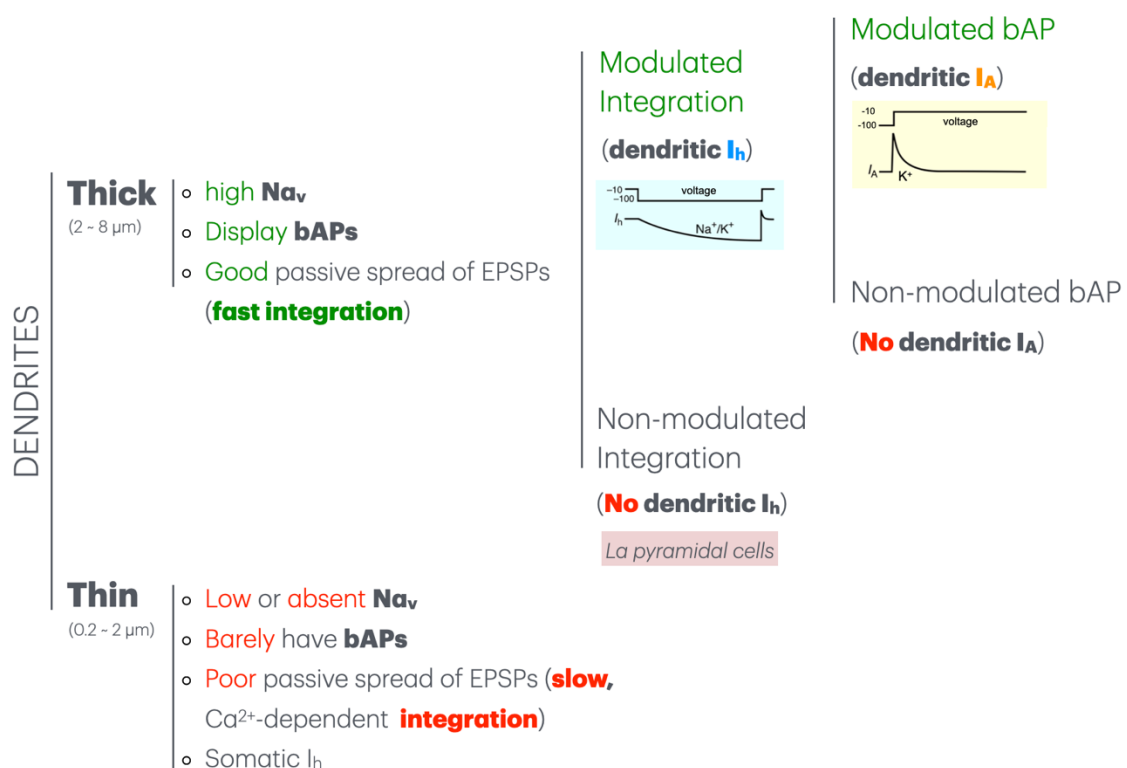


Figure 5.2.3. Separation of neurons by biophysical parameters. One of the most important parts of neurons are dendritic trees, where most relevant computations are now thought to occur. Dendrite diameters vary considerably from very thin (0.2 – 2 μm) to relatively thick (2 – 8 μm). Very interestingly, the latter determine to a large extent how fast the trees integrate incoming signals: while thick dendrites display elevated numbers of voltage-gated sodium channels (Na_v), which allow backpropagation of action potentials (bAPs) and good passive spread of EPSPs, thin dendrites barely have Na_v 's and bAPs, and their poor EPSP spreading is mostly driven by slower calcium currents instead. Signal integration by thick dendrites may be modulated by the presence of hyperpolarization-activated cyclic nucleotide-gated (HCN) channels, which allow I_h inward currents to influence how easily EPSPs buildup. Pyramidal-like cells from the amygdala La nucleus seem to be devoid of HCN channels in their dendrites, which are therefore non-modulated integrators. Finally, bAPs may also be modulated by the presence of voltage-gated A-type potassium channels (K_{vA}), which generate outward I_A currents. Image based on Migliore & Shepherd (2005) and Byrne *et al.* (Ed.) (2014).

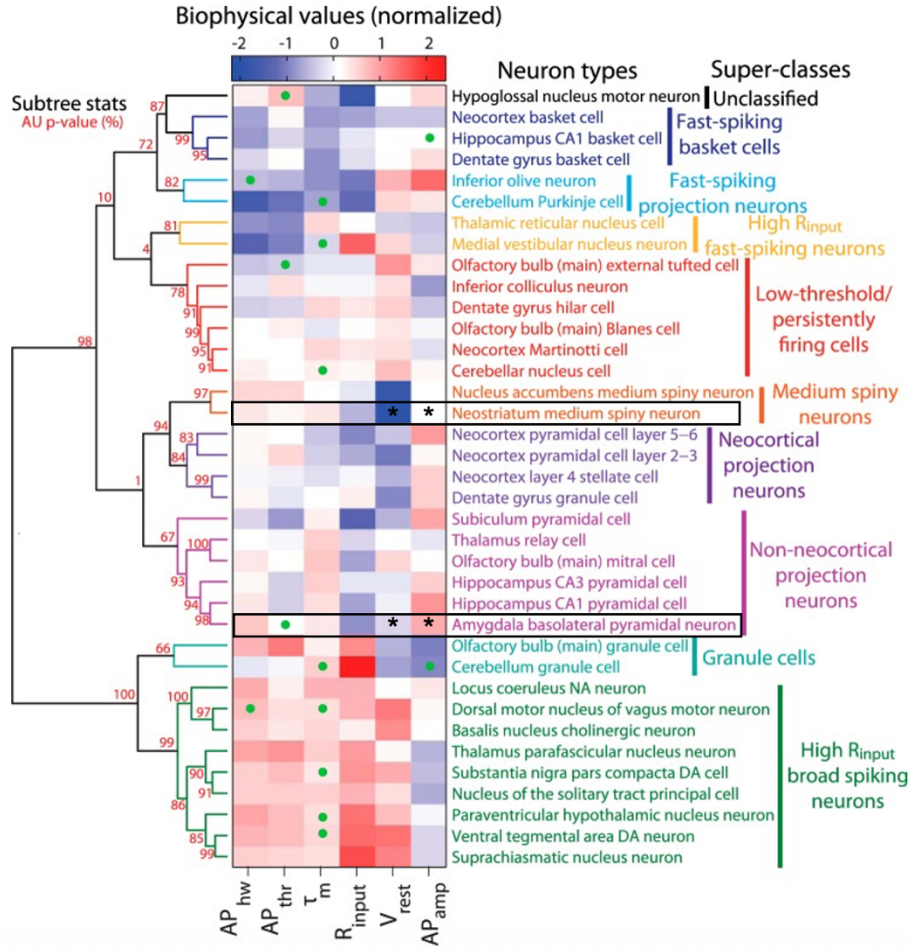


Figure 5.2.4. Neuron clustering based on their shared biophysical properties. Heatmap values represent neuron type-specific electrophysiological measurements, with red/blue indicating larger/smaller normalized values. Two neuron types of interest are highlighted with black rectangles. Asterisks (*) denote most dissimilar parameters (see main text). Green dots indicate imputed unobserved measurements. AP_{hw} , half-width action potential; AP_{thr} , AP threshold; τ_m , membrane time constant; R_{input} , input resistance; V_{rest} , resting potential; AP_{amp} , AP amplitude; AU, approximately unbiased. Reproduced with minor modifications from Tripathy et al. (2015).

distribution would be a logical move in setting up neuron identity (**Figure 5.2.3**). Analysis of a large group of neuronal dendritic trees shows that their dendrites can be broadly separated into **thick** or **thin**, that is, of wider ($2 \sim 8 \mu\text{m}$) or thinner ($0.2 \sim 2 \mu\text{m}$) diameters, respectively. In the hippocampus, for example, CA1 pyramidal interneurons fall into the first category, while CA3 pyramidal interneurons fall into the second. This difference might seem at first unimportant, but it has several neurocomputational implications. Smaller dendritic diameters underlie higher axial resistances, which promote faster decay of EPSP spreading along dendrites. On the other hand, thicker dendrites correlate with high expression of dendritic voltage-gated sodium (Na_v) channels, allowing for faster Na^+ -dependent integration and display of bAPs. Thinner dendrites barely have any Na_v channels, instead displaying

slow Ca^{2+} -dependent integration and essentially no bAPs (Migliore & Shepherd, 2005)³¹. We see that not all pyramidal cells are alike in their dendritic channel distributions and respective integrative abilities, even within the same local region, such as e.g. the hippocampus.

Due to huge variability across reports, some authors have used literature-based approaches (meta-analysis via statistical methods), centered on electrophysiological data only (here from rats, mice, and guinea-pigs), to try to overly classify neurons into different super-classes (Tripathy et al., 2015) (**Figure 5.2.4**). We will not go into detail, for the sake of accuracy (e.g. there is not *one* τ_m for a neuron — this may vary depending on many factors, including animal age, firing history, type of stimulus, etc.) but as a simple overall exercise,

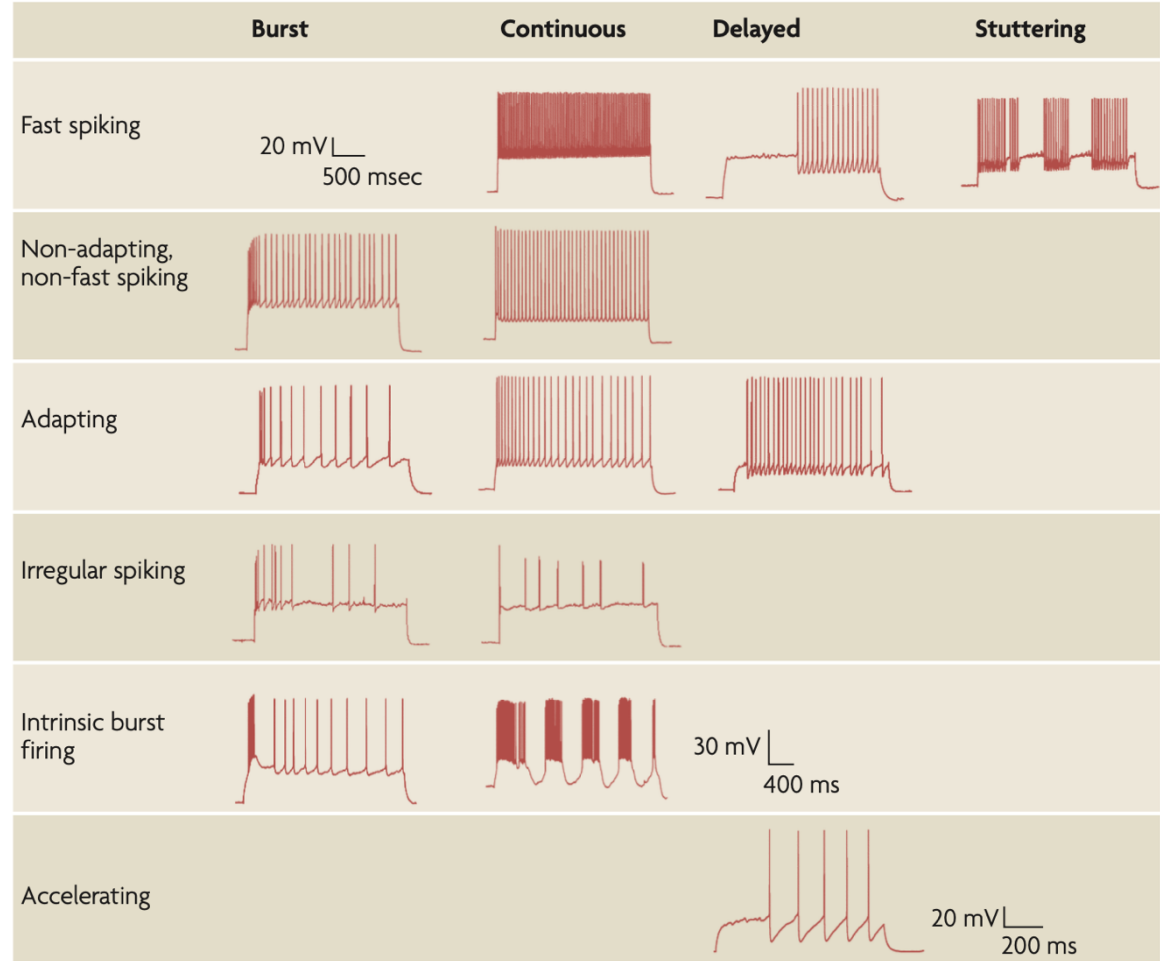


Figure 5.2.5. Types of neuron firing patterns as defined at the Petilla convention (2008). The figure illustrates some of the most common responses of cortical GABAergic interneurons to standardized intrasomatic step-current injections in the rat neocortex. Features of firing patterns in response to step-onset are organized in columns, while steady-state patterns are displayed in rows. From Petilla Interneuron Nomenclature Group et al. (2008).

³¹ Still, in most dendritic trees the relative dendritic diameter tends to decrease the more distal one gets, so that many neurons display both thick and thin dendrites. Therefore, bAPs tend to occur more proximally.

we drew a comparison between a BL neuron and a striatal medium spinal neuron (MSN) (see rectangle highlights in **Figure 5.2.4**) to show that, although having been placed into different categories, these cells are indeed electrophysiologically similar. Despite a slightly lower-than-average MSN resting potential (V_{rest}), and a higher-than-average BL AP amplitude (AP_{amp} ; see figure asterisks), both neuron types display quite parallel biophysical tendencies, which might indicate similar origins or functional properties. The reader may further observe the schematic in search for other surprising similarities. Further to these insights from biophysical data, computational methods that allow simulation of electrical properties from *in vitro* data have become increasingly common and are quite accurate when comparisons are made with *in vivo* data. In a very interesting report from the Blue Brain Project (2015), the authors reconstructed an S1 neocortical volume of 0.29 ± 0.01 mm³ containing ~31000 neurons and identified what they called 207 *morphoelectrical* neuron subtypes (Markram et al., 2015), in which an attempt is made to cross cell morphologies with electrophysiological characterization via patch-clamp recordings, taking on neuron firing patterns as described by an extended version of the one proposed at the Petilla convention (Petilla Interneuron Nomenclature Group et al., 2008) (**Figure 5.2.5**). Although much research work is still ahead in this area, such as within less-accessible subcortical structures (e.g. amygdala nuclei), these first drafts show promise in combining structure and function as main characterizers of neuron identity. For the amygdala in particular, we are yet at the molecular stage, but with important contributions to the identification of genetic predictors of neuronal involvement in fear conditioning encoding and expression (Hochgerner et al., 2023). Let's hope some day activity patterns will be part of that picture.

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