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The hypothetical utilisation of Entomopoxvirus to achieve rejuvenation through cyclic expression of Yamanaka factors

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ABSTRACT

Epigenetic reprogramming using cyclic expression of Yamanaka factors has been demonstrated to rejuvenate on a systemic level in animal models with no obvious deleterious effects.
Here we discuss a strategy for cyclic and systemic expression of Yamanaka factors in wild-type animals amenable for adaptation as a human therapy. This is based on the use of the little-known Amsacta moorei Entomopoxvirus vector in combination with microbubble mediated focused ultrasound-driven blood vessel permeabilization to facilitate the system-wide distribution of the virus.

1. Motivating Prior Art

While multiple interventions capable of extending life span and health span have been identified in animal models, few of these have been successfully translated into therapies with proven efficacy in humans. This reflects at least in part that the most effective treatments in animal models involve drugs with deleterious effects, genetic modifications or the specific silencing of genes, while human interventions are limited to dietary interventions and well-tolerated drugs. One recent approach which has proven efficacious in animal models exploits the phenomenon occurring in the creation of induced pluripotent cells where the epigenetic programming of such cells appears to revert to a more youthful state. It has been demonstrated in cell culture that the same reprogramming effect can be achieved by a short transient expression of the Yamanaka factors used to create iPSCs but with the retention of cellular identity [1].

Moreover a strong cumulative effect has been demonstrated using cyclic gene expression of the Yamanaka factors (OSKM) which has wide-ranging rejuvenation effects both in vitro and in vivo [1]. Importantly, if expression levels are suitably controlled this can be achieved in vivo without the formation of teratomers or other fatal effects [1].

This was demonstrated in an experiment that involved 2 mouse lines both expressing the OSKM systemically under the control of doxycycline. Importantly one of these mouse lines was based on a wild type mouse and was allowed to age naturally before being exposed to doxycycline cyclically 2 days out of 7 to induce cyclic OSKM expression. Treated aged animals demonstrated a rejuvenated profile of regenerative ability in response to chemical damage to their pancreas and muscles. Fibroblasts extracted from these mice and cultured in vitro showed reduced markers for DNA damage, ROS, senescence and epigenetic dysregulation. Interestingly late-passage human fibroblasts engineered to cyclically express OSKM were also tested, giving similar results. Unfortunately, applying this to human therapy is limited by the fact that systemic gene therapy across multiple tissue types is usually synonymous with germline gene therapy.

It has also been shown the amsacta moorei entomopoxvirus (AmEPV) endogenous to moths and butterflies can act as a gene therapy vector that achieves transient gene expression of a small subset of its genes for less than 24 hours [2]. This vector abortively infects most mammalian cell types without any known or apparent cytotoxic effects or inhibition of cell division: Since the virus localises to the cytoplasm (it's normal place of replication) the cell's stage of cell division is irrelevant.

The virion enters the cell by exploiting glycosaminoglycans which are widely expressed across cell types and species [3]. After entry, the virion remains in the cytoplasm and initially only partially uncoats. It's DNA is confined within the virion and an RNAP packaged with the virion produces mRNA that facilitates later stages of its reproduction. In mammal cells, the virion is unable to uncoat, and it's RNAP will cease to function, leaving the virion essentially an inert mass of protein in the cytoplasm.

These virions are relatively large and most likely reliant on infecting and budding from endothelial cells to spread systemically. Given AmEPV is native to Lepidoptera it's unlikely to have any surface markers that would facilitate transport across human endothelium. Combined with its abortive infection of human cells AmEPV virion are unlikely to pass through normal blood vessel walls.

Recent developments in localised gene therapy have demonstrated the use of microbubbles and intense focused ultrasound to create targeted permeability of the blood-brain barrier [4]. This technique utilizes adeno-associated viruses (AAV), normally unable to penetrate the blood vessel wall, as well as microbubbles, a micro-sized bubble of usually lipid/polymer bound dense non-toxic gas, and has been shown to involve disruption of the adhesive junction between

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endothelial cells [4, 5]. More intense ultrasound has been shown to correspond to larger gaps in the blood vessel wall and greater gene expression in the tissue, as indicated by transmission electron micrographs of blood vessel walls and AAV-GFP induced fluorescence in treated tissue [4]. The microbubbles used (SonoVue SF6) are already approved for clinical use and human studies on healthy volunteers suggest a daily dose twice as high as those used in Hsu's study would be safe [4, 5, 6]. The principal risk of microbubbles is thought to be reduced blood oxygenation, and lung function as SF6 from the microbubbles escapes from the lungs. However, for both healthy volunteers and patients with COPD, no statistically significant effect was observed compared to placebo (although subjects with COPD received a lower dose) [6].

2. Proposal

Here, we propose that cyclic expression of OSKM in standard mouse lines such as C57BL/6 could be achieved without prior genetic engineering by the combination of ultrasound, microbubbles, and AmEPV. This would have several advantages over previous techniques. In particular, exposing different body parts to varying intensities of ultrasound would allow the intensity of OSKM expression to be tailored to different tissue types to balance the risk of dedifferentiation against therapeutic effectiveness on a location-by-location basis.

Construction of a single AmEPV vector capable of carrying all 4 OSKM genes likely requires the creation of an engineered line of gypsy moth cells (LD-652). AmEPV has 2 redundant genes (for replication in LD-652 cells), which can be overwritten with transgenes. However, addition of native AmEPV genes (such as the iap gene) to the LD-652 genome would make them redundant for AmEPV replication [7, 8]. In this way, an AmEPV vector causing expression of the 4 OSKM genes in most mammalian cells could be constructed. Alternatively, two strains of AmEPV, each expressing half of the OSKM genes, could be used. Either way, synthesis of the vectors with the newly inserted transgenes has been well described, although more modern methods might streamline the process [7, 9].

We propose the AmEPV gene vector could be mixed with suitably sized microbubbles. The vector could then be injected into an anesthetized geriatric mouse, so chosen to facilitate examination of any rejuvenation effects vs controls. The mouse being clamped in a chamber can be scanned with a highly focused ultrasound beam for which intensity is set according to a pre-programmed pattern. This would allow varied control of gene expression intensity in different organs. A GFP based control could be used to verify successful gene expression in the experiment.

The delivery of the AmEPV gene vector procedure would be repeated, most likely once a day for 2 consecutive days out of each week, in line with the cycle previously described in literature and the roughly 20-24h expression period for AmEPV [1, 2]. The mouse's age-related phenotype can be characterised using clinically relevant longitudinal multiple parameter frailty index testing, with further tests to quantify cognitive and neuromuscular performance [10, 11, 12]. This assessed at regular intervals and be interspaced with the treatment protocol. At the study end-point mice can be sacrificed for histological analysis of tissues and changes in factors such as cytokines, organ function and senescence cell burden.

Late-adult mice can be purchased directly at 18 months from suppliers such as Charles River, although this is costly and mice may need to be kept for a further 12 or more months to monitor health and lifespan. Alternatively, accelerated ageing can be induced systemically in young mice using insults such as radiotherapy [12] and chemotherapy [13]. Alternatively, damage can be caused in specific organs with targeted treatment [14, 15], or with disease-mimicking insults such as carbon tetrachloride induced liver fibrosis [16].

Notably, inducing permanent expression of Oct4, Sox2 and Klf4 in retinal ganglion cells has been shown to recover their youthful regenerative potential [17]. However, such schemes generally rely on using a subset of the Yamanaka factors. Given that the Yamanaka factors are themselves naturally expressed in isolation in certain tissues, this would seem to preclude system-wide induced expression of a subset of the Yamanaka factors to avoid co-expression with the omitted naturally expressed Yamanaka factors. In particular, c-myc is expressed in many proliferating cells, which might be why the systemic expression of OSK still leads to teratoma formation [18, 19, 20].

Pox viruses are not the only means of transient expression of Yamanaka factors in cells. Engineered virus-like particles (VLP) might deliver the transcription factors or their mRNA to cells [21, 22]. VLPs tend to be quite immunogenic and typically have a strong tropism for certain cell / tissue types [23, 22]. However, non tissue-specific targeting is plausible, for example, MS2 VLPs bearing the HIV-1 Tat(47-57) peptide have been shown to deliver RNA to multiple cell types and organs, and the Tat peptide has a non-cell receptor dependent method of entry [24]. Some work towards reducing the immunogenicity of VLPs has also been done, for example by binding PEtOx to hepatitis B core VLPs [25]. Engineered virus-like particles might prove a good vector for cyclic OSKM therapy in the future but is arguably currently underdeveloped for that kind of use.

The key concern in moving the technique we describe toward medical therapy is that it may induce the formation of teratomas or other dysplasia. This is what is seen in permanent or long-term (on order of 1 week plus) expression of Yamanaka factors, all be it in a slightly different mouse model [26]. Even here, shorter more intense expression (1 week at 1mg/ml doxycycline vs 2.5 weeks 0.2mg/ml) seems to give rise to fewer teratomas, so it shouldn't be so surprising that the short 2 out of 7 day cycle, which shows no evidence of teratomas, seems safe in the system described by Ocampo [1, 26]. Whether this holds true in this proposed system of expression is one of the key questions

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to be answered by the proposed experiment, but with the precise temporal and spatial control, we believe this method of delivery is most likely to yield successful results in mice, and have the greatest potential for translation to human therapy

3. Discussion

The number of strains required to express OSKM in tissue is potentially dependent on its means of production. AmEPV has a large genome that could easily hold the 4 genes many times over [2]. However only two of its genes are known to be redundant for replication in moth cell culture [2]. To the best of my knowledge, no system for creating AmEPV particles with entirely arbitrary DNA contents exists, but such a system could be developed. In particular, it would be fairly straightforward to genetically engineer the cells normally used to culture AmEPV to constitutively express several of AmEPVs early phase genes, making them redundant in the virus itself.

It should also be noted that an additional crude level of gene expression control can be achieved by promoter selection. AmEPV has two promoters that lead to transcription in mammalian cells, but one leads to significantly stronger expression than the other [2].

It's also conceivable that the AmEPV particle will not provoke an innate immune reaction. The leading theory as to why the AmEPV particle is unable to replicate in mammalian cells is that it never fully uncoats [2]. This leaves its DNA sequestered in the viral particle, where it is likely only visible to the transcription machinery packaged inside the viral particle itself. In the absence of naked DNA, it's quite possible the virus won't trigger cells' innate viral response [27].

The closest human infecting relatives of AmEPV are smallpox and monkeypox, neither of which the average person has any prior exposure to. It's therefore also possible that given it's lack of pathogenicity, AmEPV will not provoke a reaction from the adaptive immune system [2]. Ofcourse it remains to be seen if the 2022 monkypox outbreak will create a large group of people with immune systems primed to react against pox viruses.

Researchers estimate of the time span of gene expression using AmEPV as being between 20 and 24 hours [2]. However, should it prove to be shorter, 3 applications over 2 days might be necessary instead of 2.

Research does not appear to have addressed how long the benefits of cyclic OSKM expression last once therapy is withdrawn, except that in a mouse model of progeria, it was observed that visual cell markers of progeria returned gradually after therapy was halted [1].

Even if therapy had to be continued indefinitely, the relatively non-invasive nature of the procedure and the fact it is required only on a few days per week may make it viable. After all, people are already willing to undergo regular Botox injections and spend regular time in sun beds to achieve desired cosmetic effects.

The size of the microbubbles may prove to be significant, with larger bubbles leading to stronger expression and presumably larger gaps in blood vessel walls [4, 5].

Although the AmEPV particle is large, the microbubble is even larger and as some less potent methods of gene expression involve using the microbubbles themselves to carry DNA, it may be supposed that at least some microbubbles pass through the blood vessel wall [5].

The use of AmEPV particles as a gene therapy vector is subject to a patent that has lapsed [28]. The advantage being that this technology may be more cheaply exploited. The disadvantage being there is no apparent commercial development of the vector, meaning it's likely any AmEPV vector must be 'home brewed' by the researcher.

It's worth considering why you would wish to use AmEPV instead of baculoviruses which are more widely used as a vector and have also demonstrated transient gene expression in mammalian cells. It has been speculated that the immune response to baculoviruses is due to naked DNA with a high level of unmethylated CpG. Conversely, inactivated virus particles provoke no response [29]. Moreover, gene expression in mammalian cells occurs in the nucleus and requires modifying the viral DNA with mammalian transcription promoters [30]. This creates cell type specific variations in gene activity as different cell types favour different promoters. It also raises the possibility of accidental incorporation of the OSKM genes into nuclear DNA, leading to teratomas.

Lastly, the typical expression period of a baculovirus in a mammalian cell is 7-14 days. More than long enough for dedifferentiation to occur [30].

It's unclear what effect repeated permeabilization of the majority of the circulatory system will have however, the effect is believed to be short-lived and it's conceivable that by the end of an ultrasonic 'scan' the areas treated at the start of the scan may already be largely closed.

Because the blood vessel walls of the blood brain barrier are the toughest in the body it's likely ultrasound gene therapy will work anywhere there is a blood supply.

Factors likely to give rise to differing gene expression across body areas are, the intensity of ultrasound used in a given area, the cell types in that area, the robustness of blood vessel walls in that area. Hopefully, with sufficient refinement, the former can compensate for the latter. Only tissue with negligible blood supply, such as the cornea and lens, are likely to be impossible to reach with this method.

One potential concern is that vaccena virus has been demonstrated to abortively infect dendritic cells suppressing their role in the immune response. It's unknown if the distantly related entomopoxvirus (EPV) would have a similar effect [31]. However, dendritic cells are relatively short-lived, regularly replenished and not required for an immune response to a previously encountered pathogen. Such a mechanism would further reduce the likelihood of immune rejection of the viral vector. One particular concern in animal studies is whether a given candidate therapy may affect cognition in a way that might not be obvious in

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animals. To that end, we propose a study of this kind should make use of cognitive testing of the subjects using video game systems integrated into their cages [32].

Given the cardiovascular system acts as the route of delivery for EPVs one might be concerned as to whether endothelial cells of the cardiovascular system will experience a disproportionate level of gene expression. It's difficult to be certain, however, EPV entry to the cell is believed to be mediated by glycosaminoglycans, membrane bound sugars that promote cell to cell and cell to ECM adhesion.

Glycosaminoglycans on the apical surface of endothelial cells form part of the glycocalyx. An unusually thick layer of sugars that helps buffer endothelial cells from the blood and keep their surface slippery. It's not clear whether EPVs will penetrate this thicker layer as easily. Human life span has increased markedly in the last century. For many people, that means they spend a larger part of the tail end of their lives living in infirmity or disease. Viable rejuvenation technology offers the hope of a kinder world where youth is prolonged into old age. Something of immense benefit both personally to individuals and society at large, which is struggling to care for its elderly.

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References

[1] A. Ocampo, P. Reddy, P. Martinez-Redondo, A. Platero-Luengo, F. Hatanaka, T. Hishida, M. Li, D. Lam, M. Kurita, E. Beyret, T. Araoka, E. Vazquez-Ferrer, D. Donoso, J. L. Roman, J. Xu, C. Rodriguez Esteban, G. Nuñez, E. Nuñez Delicado, J. M. Campistol, I. Guillen, P. Guillen, J. C. Izpisua Belmonte, In vivo amelioration of age-associated hallmarks by partial reprogramming., Cell 167 (2016) 1719–1733.

[2] Y. Li, R. L. Hall, R. W. Moyer, Transient, nonlethal expression of genes in vertebrate cells by recombinant entomopoxviruses, Journal of Virology 71 (1997) 9557–9562.

[3] B. Moss, Membrane fusion during poxvirus entry, Seminars in Cell & Developmental Biology 60 (2016) 89–96.

[4] P.-H. Hsu, K.-C. Wei, C.-Y. Huang, C.-J. Wen, T.-C. Yen, C.-L. Liu, Y.-T. Lin, J.-C. Chen, C.-R. Shen, H.-L. Liu, Noninvasive and targeted gene delivery into the brain using microbubble-facilitated focused ultrasound, PLOS ONE 8 (2013) e57682–.

[5] C. Huang, H. Zhang, R. Bai, Advances in ultrasound-targeted microbubble-mediated gene therapy for liver fibrosis., Acta Pharm Sin B 7 (2017) 447–452.

[6] D. Bokor, J. B. Chambers, P. J. Rees, T. G. Mant, F. Luzzani, A. Spinazzi, Clinical safety of sonovue, a new contrast agent for ultrasound imaging, in healthy volunteers and in patients with chronic obstructive pulmonary disease., Invest Radiol 36 (2001) 104–109.

[7] A. Cid-Arregui, A. . X. García-Carrancá, Viral vectors: basic science and gene therapy, Eaton Publishing Company, 2000.

[8] Q. Li, P. Liston, R. W. Moyer, Functional analysis of the inhibitor of apoptosis (iap) gene carried by the entomopoxvirus of amsacta moorei., J Virol 79 (2005) 2335–2345.

[9] R. S. Noyce, S. Lederman, D. H. Evans, Construction of an infectious horsepox virus vaccine from chemically synthesized dna fragments, PLOS ONE 13 (2018) e0188453–.

[10] M. B. Schultz, A. E. Kane, S. J. Mitchell, M. R. MacArthur, E. Warner, D. S. Vogel, J. R. Mitchell, S. E. Howlett, M. S. Bonkowski, D. A. Sinclair, Age and life expectancy clocks based on machine learning analysis of mouse frailty., Nat Commun 11 (2020) 4618.

[11] A. E. Kane, S. N. Hilmer, D. Boyer, K. Gavin, D. Nines, S. E. Howlett, R. de Cabo, S. J. Mitchell, Impact of longevity interventions on a validated mouse clinical frailty index., J Gerontol A Biol Sci Med Sci 71 (2016) 333–339.

[12] E. Fielder, T. Wan, G. Alimohammadiha, A. Ishaq, E. Low, B. M. Weigand, G. Kelly, C. Parker, B. Griffin, D. Jurk, V. I. Korolchuk, T. von Zglinicki, S. Miwa, C. Isales, Short senolytic or senostatic interventions rescue progression of radiation-induced frailty and premature ageing in mice, eLife 11 (2022) e75492.

[13] M. Demaria, M. N. O'Leary, J. Chang, L. Shao, S. Liu, F. Alimirah, K. Koenig, C. Le, N. Mitin, A. M. Deal, S. Alston, E. C. Academia, S. Kilmarx, A. Valdovinos, B. Wang, A. de Bruin, B. K. Kennedy, S. Melov, D. Zhou, N. E. Sharpless, H. Muss, J. Campisi, Cellular senescence promotes adverse effects of chemotherapy and cancer relapse., Cancer Discov 7 (2017) 165–176.

[14] A. Chandra, A. B. Lagnado, J. N. Farr, D. G. Monroe, S. Park, C. Hachfeld, T. Tchkonia, J. L. Kirkland, S. Khosla, J. F. Passos, R. J. Pignolo, Targeted reduction of senescent cell burden alleviates focal radiotherapy-related bone loss., J Bone Miner Res 35 (2020) 1119–1131.

[15] E. Fletcher-Sananikone, S. Kanji, N. Tomimatsu, L. F. M. Di Cristofaro, R. K. Kollipara, D. Saha, J. R. Floyd, P. Sung, R. Hromas, T. C. Burns, R. Kittler, A. A. Habib, B. Mukherjee, S. Burma, Elimination of radiation-induced senescence in the brain tumor microenvironment attenuates glioblastoma recurrence., Cancer Res 81 (2021) 5935–5947.

[16] Y.-l. Bao, L. Wang, H.-t. Pan, T.-r. Zhang, Y.-h. Chen, S.-j. Xu, X.-l. Mao, S.-w. Li, Animal and organoid models of liver fibrosis, Frontiers in Physiology 12 (2021).

[17] Y. Lu, B. Brommer, X. Tian, A. Krishnan, M. Meer, C. Wang, D. L. Vera, Q. Zeng, D. Yu, M. S. Bonkowski, J.-H. Yang, S. Zhou, E. M. Hoffmann, M. M. Karg, M. B. Schultz, A. E. Kane, N. Davidsohn, E. Korobkina, K. Chwalek, L. A. Rajman, G. M. Church, K. Hochedlinger, V. N. Gladyshev, S. Horvath, M. E. Levine, M. S. Gregory-Ksander, B. R. Ksander, Z. He, D. A. Sinclair, Reprogramming to recover youthful epigenetic information and restore vision, Nature 588 (2020) 124–129.

[18] K. Ohnishi, K. Semi, T. Yamamoto, M. Shimizu, A. Tanaka, K. Mitsunaga, K. Okita, K. Osafune, Y. Arioka, T. Maeda, H. Soejima, H. Moriwaki, S. Yamanaka, K. Woltjen, Y. Yamada, Premature termination of reprogramming in vivo leads to cancer development through altered epigenetic regulation., Cell 156 (2014) 663–677.

[19] A. Wilson, M. J. Murphy, T. Oskarsson, K. Kaloulis, M. D. Bettess, G. M. Oser, A.-C. Pasche, C. Knabenhans, H. R. Macdonald, A. Trumpp, c-myc controls the balance between hematopoietic stem cell self-renewal and differentiation., Genes Dev 18 (2004) 2747–2763.

[20] D. Morello, C. Asselin, A. Lavenu, K. B. Marcu, C. Babinet, Tissue-specific post-transcriptional regulation of c-myc expression in normal and h-2k/human c-myc transgenic mice., Oncogene 4 (1989) 955–961.

[21] A. Gutkin, D. Rosenblum, D. Peer, Rna delivery with a human virus-like particle, Nature Biotechnology 39 (2021) 1514–1515.

[22] S. Banskota, A. Raguram, S. Suh, S. W. Du, J. R. Davis, E. H. Choi, X. Wang, S. C. Nielsen, G. A. Newby, P. B. Randolph, M. J. Osborn, K. Musunuru, K. Palczewski, D. R. Liu, Engineered virus-like particles for efficient in vivo delivery of therapeutic proteins, Cell 185 (2022) 250–265.e16.

[23] S. Nooraei, H. Bahrulolum, Z. S. Hoseini, C. Katalani, A. Hajizade, A. J. Easton, G. Ahmadian, Virus-like particles: preparation, immunogenicity and their roles as nanovaccines and drug nanocarriers, Journal of Nanobiotechnology 19 (2021) 59.

 doi:10.20944/preprints202209.0401.v1

[24] Y. Pan, Y. Zhang, T. Jia, K. Zhang, J. Li, L. Wang, Development of a microrna delivery system based on bacteriophage ms2 virus-like particles, The FEBS Journal 279 (2012) 1198–1208.

[25] S. Y. Fam, C. F. Chee, C. Y. Yong, K. L. Ho, A. R. Mariatulqabtiah, H. Y. Lau, W. S. Tan, Shielding of hepatitis b virus-like nanoparticle with poly(2-ethyl-2-oxazoline), International Journal of Molecular Sciences 20 (2019).

[26] M. Abad, L. Mosteiro, C. Pantoja, M. Cañamero, T. Rayon, I. Ors, O. Graña, D. Megías, O. Domínguez, D. Martínez, M. Manzanares, S. Ortega, M. Serrano, Reprogramming in vivo produces teratomas and ips cells with totipotency features, Nature 502 (2013) 340–345.

[27] R. W. Moyer, Y. Li, R. L. Hall, Entomopoxvirus-vertebrate gene delivery vector and method, 2000. US Patent 6,106,825A.

[28] R. W. Moyer, Y. Li, Materials and methods for delivery and expression of heterologous dna in vertebrate cells, 2000. US Patent 6,127,172.

[29] C. Ono, T. Okamoto, T. Abe, Y. Matsuura, Baculovirus as a tool for gene delivery and gene therapy, Viruses 10 (2018).

[30] S. Schaly, M. Ghebretatios, S. Prakash, Baculoviruses in gene therapy and personalized medicine., Biologics 15 (2021) 115–132.

[31] J. Engelmayer, M. Larsson, M. Subklewe, A. Chahroudi, W. I. Cox, R. M. Steinman, N. Bhardwaj, Vaccinia virus inhibits the maturation of human dendritic cells: a novel mechanism of immune evasion., J Immunol 163 (1999) 6762–6768.

[32] A. E. Horner, C. J. Heath, M. Hvoslef-Eide, B. A. Kent, C. H. Kim, S. R. O. Nilsson, J. Alsiö, C. A. Oomen, A. Holmes, L. M. Saksida, T. J. Bussey, The touchscreen operant platform for testing learning and memory in rats and mice, Nature Protocols 8 (2013) 1961–1984.